An investigation of passive antibody and its effects on porcine epidemic diarrhea virus infection

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology (Preventive Medicine)

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The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

DEDICATION

To my parents

Dr. Kriengsak and Arunee Poonsuk

To my major professor

Professor Dr. Jeffrey J. Zimmerman

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ABSTRACT

The objectives of this research were to determine the effects of maternally-derived antibodies in protecting piglets against porcine epidemic diarrhea virus (PEDV). In Chapter 3, a passive antibody transfer model was used to evaluate the impact of circulating antibody on the protection of naïve piglets against PEDV. Piglets derived from 6 sows were randomly assigned to receive one of 6 different levels of concentrated serum antibody. In Chapter 4, PEDV-immune and PEDV-negative sows were used to evaluate the impact of lactogenic antibody on the protection of piglets against PEDV. In both studies, piglets were inoculated with PEDV and monitored for 14 days, during which time they remained with their dam. Sow milk, piglet fecal samples, and data on piglet clinical signs, body weight, and body temperature were collected daily. Serum, colostrum, and milk were tested for PEDV antibody by PEDV IgG and IgA ELISAs and by fluorescent focus neutralization (FFN) assay. The analysis showed that both passive antibody and lactogenic antibody contributed to the protection of the neonatal piglets against PEDV infections. Therefore, the optimal protection to piglets will be provided by dams able to deliver sufficient lactogenic immunity, both humoral and cellular, to their offspring.

The feasibility of improving oral fluid IgG and/or IgA antibody testing by clarifying samples using chemical treatment was evaluated in Chapter 5. Three chemical formulations based on chitosan and/or Tween-20® were used to treat aliquots of known status oral fluid samples from PEDV-inoculated pigs. All aliquots were tested by PEDV IgG and IgA ELISAs on day post-treatment (DPT) 0, then kept at 4°C and re-tested on DPT 2, 4, and 6. All formulations went into solution quickly and easily upon addition of oral fluid and the addition of chitosan was shown to effectively clarify oral fluids. Statistical analysis found that neither chitosan nor Tween-20® adversely affected the ELISA results and the treated oral fluid samples were stable over time. Thus, this study found that chitosan could be used to efficiently clarify oral fluid specimens without affecting the results of antibody ELISAs.

CHAPTER 1. GENERAL INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) has caused significant morbidity and mortality in neonatal pigs since its introduction into the Western Hemisphere in 2013 (Chen et al., 2014). Control of PEDV is complicated by the fact that the virus most severely affects neonatal pigs; a stage in the lifecycle when the immune system of the piglet is underdeveloped. That is, piglets are born agammaglobulinemic with limited cell-mediated immunity and do not become fully immunologically competent until about four weeks of age. This means that their limited ability to respond to infectious agents during the first month of life must be supplemented by maternal immune components, in particular, passive antibodies and immune cells provided in mammary secretions.

The role of maternal immunity in the protection of neonates against enteric disease was first recognized through research on transmissible gastroenteritis virus (TGEV) (Bohl and Saif, 1975; Saif et al., 1972). When applied to the prevention and control of PEDV, the same principles apply: passive immunity, i.e., PEDV IgG and secretory (S)-IgA in mammary secretions is central to limiting the infection of PEDV in the neonatal intestinal tract and protecting piglets against clinical diseases (Pensaert, 1999). Manipulating sow herd immunity against PEDV through exposure or vaccination is a useful approach for managing and/or delaying infection until the piglets' immune system is better prepared to respond, but to a large extent, central questions regarding PEDV lactogenic immunity have not been addressed. Therefore, the contribution of passive antibody and lactogenic antibody were evaluated in Chapter 3 and 4 of this dissertation.

Oral fluid-based monitoring is a promising, if still unproven, method for monitoring lactogenic immunity. For PEDV diagnoses, Bjustrom-Kraft et al. (2016) found that both PEDV IgG and IgA antibody were detectable in oral fluid samples by 13 days post-exposure, but the IgA response was more robust and of longer duration than the IgG response. Notably, the oral fluid IgA ELISA sample-to-positive (S/P) ratios continued to increase for ~90 days post exposure. However, the oral fluid samples collected in the field routinely contain fine particulates, i.e., feces, soil, and feed particles, that potentially impact pipetting

accuracy and/or test performance. Particularly as laboratories move to higher-throughput technologies, e.g., robotics, it may be desirable to standardize the condition of oral fluid samples. "Clarification" using a chemical coagulant is an approach that can be applied to of swine oral fluid samples. Immediate effect (day post treatment, DPT 0) and residual effect (DPT 2, 4, 6) of the treatment on the diagnostic performance of PEDV IgG and IgA ELISAs were evaluated in Chapter 5 of this dissertation.

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CHAPTER 2. HISTORICAL AND CONTEMPORARY ASPECTS OF MATERNAL IMMUNITY IN SWINE

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Modified from a manuscript submitted to Animal Health Research Reviews

Abstract

Maternal immunity plays a pivotal role in swine health and production because piglets are born agammaglobulinemic and with limited cell-mediated immunity, i.e., few peripheral lymphoid cells, immature lymphoid tissues, and no effector and memory T-lymphocytes. Swine do not become fully immunologically competent until about four weeks of age, which means that their compromised ability to respond to infectious agents during the first month of life must be supplemented by maternal immune components: 1) circulating antibodies derived from colostrum; 2) mucosal antibodies from colostrum and milk; and 3) immune cells provided in mammary secretions. Since maternal immunity is highly effective at protecting piglets against specific pathogens, strengthening sow herd immunity against certain diseases through exposure or vaccination is a useful management tool for ameliorating clinical effects in piglets and/or delaying infection until the piglets' immune system is better prepared to respond. In this review we discuss the anatomy and physiology of lactation, the immune functions of components provided to neonatal swine in mammary secretion, the importance of maternal immunity in the prevention and control of significant pathogens.

Keywords: swine, lactogenic immunity, lactation, mammogenesis, lactogenesis

Introduction

This review describes the anatomical and physiological processes in sows that result in the passive transfer of immune constituents to the neonate through the ingestion of colostrum (*colostrogenic immunity*) or milk (*lactogenic immunity*), including both cell-mediated (CMI) and humoral immune components (Saif, 1990). The lengthy history of these concepts is worthy of note. In early Rome, the words *immunitas* and *immunis* meant "exempt from a social duty", but there are also examples in which they were used to express exemption from, or resistance to, disease (Silverstein, 1989). The widespread adoption of Edward Jenner's smallpox vaccine (1774) brought the idea of “immunization” and “vaccination” into the mainstream lexicon (Silverstein, 1989). Passive immunity, the realization that the administration of immune serum could protect the naïve recipient, was brought to light in the work of Von Behring and Kitasato (Von Behring and Kitasato, 1890a). Initially, they showed that mice (n = 8) intraperitoneal inoculated with blood from rabbits with antibodies against tetanus toxin were able to survive exposure to the toxin. Later, they pioneered the use of equine antisera to treat humans for tetanus and diphtheria (Von Behring and Kitasato, 1890b). From there, it was a short step to the realization that passive immunity could be provided by a mother to her progeny. This idea was supported by clinical observations and experiments showing that disease resistance in offspring was related to the disease (or vaccination) history of the mother, as described below.

The recognition of maternal immunity

Otto Bollinger (1877) was the first to hypothesize the *in utero* transfer of maternal immunity. Initially, he had observed a case in which a newborn child from a smallpox-vaccinated mother was resistant to smallpox vaccination, i.e. the baby did not show the vesicular lesions indicative successful vaccination against smallpox. He then conducted a controlled clinical experiment and found that lambs from ewes (n = 700) vaccinated with sheep pox (*variola ovina*) showed resistance to vaccination, i.e. the lambs from vaccinated mothers did not develop the vesicular lesions if vaccinated at 4 to 6 weeks-of-age.

Shortly thereafter, Albrecht E. Burckhardt (1879), conducted a controlled clinical experiment and found that infants from mothers who had been successfully vaccinated against smallpox in late pregnancy showed resistance to smallpox vaccination, i.e., did not develop the vesicular lesions indicative of successful vaccination when inoculated at 4 to 6 days of age. Burckhardt concurred with Bollinger that the mother's immunity against smallpox was transferred to the fetus *in utero*.

In 1880, Auguste Chauveau (France) described a series of experiments that proved that lambs born from ewes inoculated during late gestation with a *Bacillus anthracis* preparation were resistant to the pathogen. Initially, Chauveau found that sheep repeatedly (7 or 8 times) inoculated with a *Bacillus anthracis* preparation at 15-day intervals stopped showing clinical signs post inoculation e.g., malaise, swollen lymph nodes, ganglionic swelling, and/or fever. Chauveau concluded that exposure no longer produced clinical signs because the sheep had produced something in response to the initial inoculations that protected them from subsequent exposures. In the next phase of his studies, he repeatedly inoculated pregnant sheep and then inoculated their lambs. Because the lambs showed no clinical signs following their first exposure, Chauveau concluded that protection could be transferred from immune mothers to their offspring (Ladnyi, 1964).

In 1892, Paul Ehrlich proved that immunity against plant toxins (abrin and ricin) could be transferred from female mice to their offspring through mammary secretions. In one experiment, he "immunized" 15 does against either abrin or ricin during pregnancy, then inoculated 22 pinkies from their litters to the same toxin. Because all pinkies survived, he hypothesized that passive immunity ("*passive immunität*") was transferred from the immune mother to their offspring through mammary secretions. To test this hypothesis, pinkies (n = 2) from immunized does were cross-fostered to a non-immunized doe and pinkies (n = 4) from non-immunized does were cross-fostered to immunized does (n = 4). None of the pinkies that nursed the non-immunized doe survived, but 3 of the 4 pinkies that suckled immunized does survived the inoculation. In addition, Ehrlich reported that the passive antibody could not be transferred from male to the offspring. Bucks (n = 6) immunized with abrin were bred with non-immunized does (n = 6), then their pinkies were inoculated with the

toxin. None of the pinkies survived and all showed the effects of the toxin e.g. intestinal hemorrhage, and tissue necrosis (Brieger and Ehrlich, 1892).

A series of studies with "*Bacillus diphtheriae*" (*Corynebacterium diphtheriae*) conducted by Theobald Smith between 1904 and 1909 provided further evidence and greater detail concerning the transfer of immunity from mother to offspring (Smith, 1905; Smith 1907; Smith 1909). Working with *Corynebacterium diphtheriae* culture fluid, Smith defined one unit of toxin as the dose able to kill one 250 to 280 gram guinea pig after subcutaneous injection and one antitoxin unit as the volume of serum able to completely neutralize one unit of toxin. In his experiments, an immune guinea pig was defined as an animal that survived a lethal dose of toxin.

Smith made four key observations:

(1.) Immunity to diphtheria toxin in the mother is transmitted to her offspring (Smith, 1905; Smith, 1907). Female guinea pigs (n = 77) were administered one of 4 treatments: no treatment (n = 21), toxin (n = 4), antitoxin (n = 7), and a toxin-antitoxin mixture (n = 35) and then bred 2 to 3 months post treatment. When their offspring weighed 250 to 280 grams, they were subcutaneously injected with a lethal dose of toxin. All guinea pigs from mothers that received the toxin-antitoxin mixture survived challenge and, over a range of challenge doses, showed greater resistance to the clinical effects of the toxin compared to other treatment groups.

(2.) Immunity to diphtheria toxin in the father is not transmitted to his offspring (Smith, 1907). Male guinea pigs (n = 6) were divided into 2 groups: treatment (n = 5) and control (n = 1). The guinea pigs in the treatment group were administered the toxin-antitoxin mixture and then bred with 11 untreated female guinea pigs. The control male guinea pig was bred with 2 untreated females. When the offspring of the female guinea pigs weighed 250 to 280 grams, they were subcutaneously administered a lethal dose of diphtheria toxin. None of the offspring survived and Smith concluded that immunity could not be transmitted from the father to the offspring.

(3.) Passive immunity does not persist in the offspring and is gradually lost over time (Smith, 1907). Offspring from 8 toxin-antitoxin treated females (n = 18) and 2 untreated females (n = 2) were subcutaneously injected with different amounts of toxin (1.0 to 1.72 units) at different ages (32 to 119 days of age) and different body weight (251 to 650 grams). The lethal dose in resistant guinea pigs gradually decreased by time such that, by 3 months of age, guinea pigs from immunized females no longer showed any resistance to the toxin. Smith observed that maternally-derived immunity did not persist; rather it gradually declined over time.

(4.) Passive immunity is not transmitted from the first generation to the second generation (Smith, 1907). Female guinea pigs ("1st generation") from immunized females were divided into 2 groups: positive control (n = 2) and untreated (n = 12). Guinea pigs in the positive control group were administered the toxin-antitoxin mixture and those in the untreated group were not. Thereafter, the offspring ("2nd generation") from both groups were administered a lethal dose of the toxin. None of the offspring from the untreated group survived whereas offspring from the positive control group were resistant to the toxin. Therefore, Smith concluded that the passive immunity was not transmitted from the first generation to the second generation.

In 1912, Lemuel William Famulener conducted an experiment to establish whether maternal antibody was transmitted from mother to offspring via mammary secretions and/or *in utero*. Female goats (n = 5) were subcutaneously inoculated with sheep red blood cells (RBC) during late pregnancy. Kids farrowed from these does were either allowed to suckle their mother (treatment group) or fed with cow's milk (control group). Specific anti-sheep RBC antibody in serum, colostrum, and milk samples from the goats and kids was measured by complement fixation. The results showed that the goats inoculated with sheep RBCs during late pregnancy secreted high concentration of anti-sheep RBC antibody in their colostrum and milk and that the kids in the treatment group showed high antibody concentration in their blood whereas no anti-sheep RBC antibody was detected in the controls, i.e., no maternal antibody had been transferred to the kids *in utero*.

Although Famulener (1912), working with goats, had concluded that the placenta was not permeable to maternal antibody, Tenbroeck and Bauer (1923) in a study of women (n = 6) known to be carriers of *Clostridium tetani*, showed that tetanus antitoxin was present in the mothers' serum, placental cord blood, and children's serum. Despite the fact that they failed to account for the colostral contribution to serum antibody in the children, they concluded that "*Since the antitoxin level in the mother's and child's blood is at approximately the same level it seems probably that the placenta is permeable to this antibody.*" McKhann and Chu (1933) went on to show that antibodies recovered from human placenta showed neutralizing activity against diphtheria toxin, group A *Streptococcus spp.*, poliovirus, and measles virus and, like Tenbroeck and Baurer (1923), concluded that neonatal immunity could have originated via transplacental transfer. Clarification was brought to the question of placental passive transfer of maternal antibody by Ladnyi (1964). Doe rabbits immunized (n = 3) with tetanus toxoid and non-immunized controls (n = 3) were bred 15 days after vaccination. After birth, the kits from the immunized females were cross-fostered to the non-immunized females and vice versa. No antitoxin antibody was detected in the serum of the offspring born from non-immunized mothers nursing immunized females, but kits from immunized mothers fed by nonimmunized females had antitoxin antibody titers approximately equivalent to their mothers'. These results supported, the conclusion that maternal antibody was transferred to fetal rabbits transplacentally.

Why are piglets born agammaglobulinemic?

In 1954, Wallace Bauriedel et al. reported that maternal antibody could not be transferred from sow to the piglet *in utero*. Cesarean-derived, colostrum-deprived piglets (n = 12) from 2 sows were housed individually and fed purified casein and soybean protein diets. Analyses of serum samples collected from the piglets over time showed that gamma globulins appeared around 6 weeks of age, i.e., no maternal antibody was provided to the piglets transplacentally.

The work of Alvin Hoerlein (1957) definitively established that maternal antibody was transferred to piglets via mammary secretions and not to fetal piglets *in utero*. A pregnant sow was subcutaneously inoculated with *Brucella abortus* (*B.abortus*) and sheep red blood cells 14 days before delivery of her piglets by cesarean section. The piglets were then divided into control piglets (n = 5) that were left with the sow to receive colostrum and colostrum-deprived piglets (n = 4) which were isolated from other pigs and fed cow's milk. Subsequent testing showed that control piglets had antibodies against both *B. abortus* and sheep red blood cells, but colostrum-deprived piglets had no detectable antibodies against either.

The explanation for why piglets are born agammaglobulinemic lies in the structure of the placenta. An initial understanding of the nature of the placenta and its role in fetal development can be traced back to the ancient Greeks, i.e., the terms "placenta", "chorion", and "amnion" were used in the treatise "*De generatione animalium*" written by Aristotle in 340 B.C. Concerning the placenta, Aristotle wrote: "*The vessels join on the uterus like the roots of plants and through them the embryo receives its nourishment*" (Longo and Reynolds, 2009). A significant understanding the mechanics of the maternal-fetal circulation within the placenta was not achieved until much later. In 1872, William Turner described the human placental structure and compared it to that of domestic animal species. In 1909, Otto Grosser described the macroscopic and microscopic diversity of mammalian placental anatomy and proposed a system of classification based on placental structure. In 1961, Emmanuel Ciprian Amoroso modified Grosser's system and developed a system of classification based on the presence of tissue layers separating the maternal and fetal blood systems: maternal endothelium, maternal connective tissue, maternal (uterine) epithelium, fetal (chorionic) epithelium, fetal connective tissue, and fetal endothelium.

(1.) Epitheliochorial placentation (swine and equids) retains all six maternal and fetal tissue layers.

(2.) Syndesmochorial placentation (ruminants) consists of five tissue layers, i.e., maternal endothelium, maternal connective tissue, chorionic epithelium, fetal connective tissue, and fetal endothelium. Thus, the maternal (uterine) epithelium is absent.

(3.) Endotheliochorial placentation (most carnivores) separates maternal and fetal blood circulation by four tissue layers including maternal endothelium, chorionic epithelium, fetal connective tissue, and fetal endothelium. Thus, the chorionic epithelium of the fetus is in direct contact with the endothelium of the maternal capillaries.

(4.) Hemochorial placentation (human and most primates) is composed of three tissue layers (chorionic epithelium, fetal connective tissue, and fetal endothelium). This brings the maternal blood circulation into direct contact with the chorionic epithelium.

(5.) Hemoendothelial placentation (most rodents and lagomorphs) is distinct in that maternal blood circulates in large sinus-like spaces in the trophoblast. Fetal capillaries projecting directly into the space are only separated from maternal blood by the fetal endothelial layer.

Both hemochorial and hemoendothelial placentation permit the transfer of maternal immunity to the fetal circulation (Leiser and Kaufmann, 1994). In these species, progeny receive passive immunity both *in utero* and via mammary secretions after birth. The endotheliochorial placentation seen in most carnivores limits the transfer of maternal immunity to the fetus and the majority of passive immunity is transferred through mammary secretions, i.e. colostrum and milk, from the mother to their offspring after birth. The six-layered epitheliochorial placenta of swine and the five-layered syndesmochorial placenta of ruminants preclude any immune transfer through the placenta due to the number of layers constituting the placental barrier (Bertasoli et al., 2015). Thus, the reason piglets are born agammaglobulinemic can be explained by the structure of the placenta.

Anatomy and physiology of lactation

Swine mammary gland anatomy

Anatomically, mammary glands in pigs are located in two parallel rows from the thoracic to the inguinal areas on the ventral body wall. Domestic pigs commonly have 14 mammary glands distributed in 3 areas of body, i.e., 4 thoracic, 8 abdominal, and 2 inguinal glands, but the total number of glands may vary between 12 and 18, depending on the genetic background of the individual (Labroue et al., 2001). Each mammary gland is usually composed of two complete gland systems i.e., the gland has two separate teat canals that connect to one nipple (Martineau et al., 2012).

The macroscopic and microscopic structures of the mammary gland differ between nulliparous and lactating sows. Mammary glands of nulliparous sows are composed of cell buds distributed among fat and connective tissue, but the tissues are displaced by glandular parenchyma in lactating sows. In lactating sows, mammary glands are composed of compound tubuloalveolar tissue forming lobular structures. The lobules are lined by epithelial cells (lactocytes) that function in milk synthesis (Klopfenstein et al., 2006), and are surrounded by myoepithelial cells that function in milk ejection and vascularized connective tissue that contains lipid-depleted adipocytes and fibroblasts (McManaman and Neville, 2003). The secreting units are connected via a ductal system to an ostium found on the end of the teat.

Mammogenesis

Mammogenesis, the development of nulliparous mammary glands into functional, lactating mammary glands, begins with the onset of puberty. Over the course of puberty, mammary gland parenchymal tissue, i.e., alveoli, ducts, and associated connective tissues, increases by 51% and the extraparenchymal tissue, i.e., adipose tissues surrounding the parenchyma, decreases by 16% (Farmer et al., 2004). Ji et al. (2006) reported that weight of swine mammary glands increased from 39.7 to 299.2 grams per gland between 45 to 112 days of gestation, reaching the maximum mammary gland alveolar epithelial cell concentration by day 90.

Mammogenesis development during pregnancy is under the influence of specific reproductive hormones, e.g., relaxin, estrogen, and prolactin, particularly during the last one-third of gestation in the pregnant gilt. In swine, estrogens (estrone and estradiol 17-β) are initially produced by embryonic blastocysts beginning at 10 to 15 days of pregnancy (Spencer and Bazer, 2004). At 17 to 19 days of gestation, fetal placental tissue becomes the primary source of estrogens (Ainsworth and Ryan, 1966; Bazer, 1977; Farmer, 2001; Knight, 1994). However, porcine myometrium and endometrium serve as supplemental sources of estradiol 17- β beginning at 14 to 16 days of gestation (Franczak and Kotwica, 2007). Relaxin is initially produced by theca interna cells of preovulation follicles during early pregnancy. Once the corpora lutea develop (10 days after ovulation), they become the major source of relaxin for the remainder of the pregnancy (Bagnell et al., 1993). Prolactin, secreted by the anterior pituitary, is essential for both mammogenesis and lactogenesis (Neville et al., 2002). Prolactin secretion during pregnancy is controlled by dopamine released by the hypothalamus. Kraeling et al. (1992) reported that prolactin concentration does not vary significantly during the first 90 days of gestation ($p < 0.05$) but increases markedly around 110 days of gestation, as discussed in Section 2.3.

Estrogen The effect of estrogens on mammogenesis is well described in swine. Sørensen et al. (2002) studied mammary development in gilts in 10 day increments from birth to day 112 of pregnancy. The rate of mammary tissue development and the DNA accumulation increased with age, but was highest after 90 days of age. The shift in mammary gland development coincided with the upward shift in estrogen production at 90 days of age. Sørensen et al. (2002) found that mammogenesis in pregnant gilts was slow during the first two trimesters of pregnancy. In the third trimester, under the influence of increasing concentrations of estrogen and progesterone and a low concentration of prolactin, the mammary glands underwent major histological remodeling, as adipose tissue and stroma were displaced by lobuloalveolar tissue and milk secretory units were formed (Kensinger et al., 1986a; Ji et al., 2006).

Kensinger et al. (1986b) created sows with different numbers of conceptuses: pseudopregnant sows (no conceptuses) were created by administration of estradiol-valerate on days 11 to 15 of the estrous cycle, 4 to 7 conceptuses by ligation of one oviduct, and 8 to 11 conceptuses by normal mating. The number of viable fetuses the sow carried was correlated with the level of serum estrogen-sulfate, which in turn reflected the wet weight, dry weight, and mammary DNA content of her mammary glands (Kensinger et al. (1986c).

Relaxin Relaxin plays a role in mammary gland parenchymal growth during the last trimester of gestation. To demonstrate the effects of relaxin on mammogenesis, Hurley et al. (1991) designed an experiment consisting of 38 gilts allocated to 3 different groups: ovariectomized gilts that received progesterone to maintain pregnancy, but no relaxin; ovariectomized gilts that received both progesterone and relaxin, and control gilts. Mammary parenchymal tissue of the gilts were measured and compared between groups on day 100 of gestation. The results showed that ovariectomized gilts that did not receive relaxin had less mammary parenchymal tissue than controls ($p < 0.05$), whereas no difference was detected in mammary parenchymal tissue between ovariectomized gilts that received progesterone and relaxin and controls ($p > 0.05$).

Prolactin The effects of prolactin on mammogenesis were reported by Farmer et al. (2000). Bromocriptine (10 mg) was orally administered 3 times daily to pregnant gilts between 70 to 110 days of gestation to inhibit prolactin production. Treated gilts and control gilts were sacrificed at 110 days of gestation. Parenchymal and extraparenchymal tissues were collected from both groups and mammary DNA, RNA, dry matter, protein, and fat content were measured. No difference was detected between the 2 groups in the weight of extraparenchymal tissue ($p \geq 0.1$), but significant decreases in the weight of parenchymal tissue, total DNA, and RNA and significant increases in fat and dry matter were observed in gilts with inhibited prolactin production ($p < 0.01$). Farmer et al. (2000) concluded that prolactin is essential for both normal mammary gland development during the last trimester of gestation and promoting lactogenesis during late gestation and after parturition (discussed in the next section).

Lactogenesis

Lactogenesis is the process by which the components of colostrum and milk are created and then secreted. Phase I of lactogenesis, beginning between 90 to 105 days of gestation, marks the synthesis of milk components (lactose, casein, and lipids) in mammary gland alveoli (Hartmann et al., 1997). Phase II, overlapping with Phase I but initiated at farrowing, involves milk synthesis and secretion initiated by progesterone withdrawal after lysis of the corpora lutea.

Phase II can be described in terms of colostral, ascending, plateau, and descending phases. The colostral phase marks the beginning of lactation immediately post farrowing (Hartmann et al., 1995). Total yield of colostrum per day is less than 50% of early lactation milk production, but colostrum contains more protein, less fat, and less sugar than milk (Dorland, 1985). In litters of 8 to 12 piglets, Hartmann et al. (1984) found that 2.5 to 5.0 (average of 3.5) kg of colostrum was produced in the first 24 hours after parturition (Hartmann et al., 1984). The ascending phase occurs 24 to 48 hours after parturition and is characterized by the switch from colostrum to milk (Klobasa et al., 1987). During the ascending phase, the frequency and volume of milk secretion increases, with maximum milk production achieved about 10 days after the switch from colostrum to milk. The plateau phase, the period that lactating sow maintain the maximum level of milk production, can last up to 28 days (King et al., 1997). The descending phase is the period of declining milk production. In modern production systems, sows usually do not reach the descending phase because piglets are weaned prior to 28 days of age (Martineau et al., 2012).

Similar to mammogenesis, lactogenesis is under the influence of hormones, e.g., prolactin, oxytocin, and growth hormone. Prolactin is responsible for maintaining milk synthesis and oxytocin stimulates the contraction of myoepithelial cells surrounding the alveolar lumen to force milk through the mammary gland ductal system to the teats (Ellendorf et al., 1982). Growth hormone plays a role in the regulation of lactogenesis by attracting nutrients necessary for the synthesis of milk components (Neville et al., 2002).

Prolactin The secretion of prolactin from the anterior pituitary and oxytocin and growth hormone from the posterior pituitary are stimulated by a neuroendocrine milk ejection reflex (Farmer, 2001; Tucker, 2000). Specifically, the physical stimulation of the piglets' suckling on the nipples sends a signal to the hypothalamus to reduce the release of dopamine into the circulation, which in turn promotes the release of prolactin by the anterior pituitary (Freeman et al., 2000). Other hormones, e.g. thyroid releasing hormone, oxytocin, and vasopressin, are other possible stimulators of the prolactin releasing reflex in pigs (Dubreuil et al., 1990; Kendall et al., 1983; Maeda and Frohman, 1978)

Research supports the crucial role of prolactin in lactogenesis. Kopinski et al. (2007) fed ergot to pregnant sows to inhibit prolactin secretion and, thereby, evaluate the effect of prolactin on lactogenesis. Pregnant sows were fed diets containing 0% (negative control), 0.3%, 0.6%, 1.2%, or 1.5% w/w of sorghum ergot sclerotia beginning 6 to 10 days before farrowing, then removed the toxin after farrowing. Sows fed 1.5% w/w ergot had lower plasma prolactin concentration compared to the controls and produced no milk. Sows fed 0.6% or 1.2% ergot showed a lesser effect on plasma prolactin reduction and milk production.

Farmer et al., (1998) evaluated the role of prolactin in the initiation and maintenance of lactation by feeding a diet containing a dopamine agonist (bromocriptine) immediately pre-farrowing or for specific periods of lactation. Gilts were assigned to 6 groups, i.e. control (n = 6), bromocriptine-contaminated feed (10 mg per 2.2 kg feed per sow per day) from 110 days of gestation until farrowing (n = 7), from 1 to 6 days postpartum (n = 6), from 7 to 13 days postpartum (n = 7), from 14 to 20 days postpartum, and from 21 to 27 days postpartum. Regardless of the stage of lactation, gilts fed bromocriptine showed significantly lower serum prolactin concentration and lower production of mammary secretions compared to the controls ($p < 0.01$) during the week of treatment.

Oxytocin During lactation, piglets stimulate the neuroendocrine reflex by physically massaging the udder, thereby causing a release of oxytocin (Algers et al., 1990). Oxytocin stimulates the contraction of myoepithelial cells surrounding the alveoli, which results in

removal of milk from the mammary gland ductal system (Fraser, 1980; Hartmann and Holmes, 1989). This stimulus-reflex response is fast, but highly transient, i.e., the increase in oxytocin concentration occurs 30 seconds before milk ejection, but oxytocin is cleared from circulation in 10 to 20 seconds (Ellendorf et al., 1982; Fraser, 1980).

Growth hormone The effect of growth hormone on lactation in swine has been demonstrated by exogenous hormone administration experiments. Initially, Harkins et al. (1989) compared milk yield in lactating sows (n = 8) administered recombinant growth hormone (porcine somatotropin, pST) at the rate of 8.22 mg per day on lactation days 12 to 29 versus untreated control sows (n = 8). Significant differences in milk composition (milk fat, milk protein, lactose, and solids) were not detected, but sows that received pST produced more milk when compared to controls ($p < 0.05$). On the other hand, Cromwell et al., (1992) compared milk production in 3 groups: controls (n = 84), sows (n = 42) that received pST (60 mg per day) from day 108 of gestation to day 24 post-farrowing, and sows (n = 42) that received pST on days 3, 10, 17, and 24 post-farrowing. In this study, it was found that sows receiving pST had a higher concentration of serum somatotropin within 4 hours after injection compared to controls ($p < 0.01$), but no difference in milk yield was detected among the 3 groups. King (2000) hypothesized that the effect of pST in lactating sows is related to demand. That is, there may be an interaction between exogenous pST and litter size such that greater milk yield is only observed in the presence of both pST and large litters with bigger piglets.

Immunological functions of mammary secretions

Cellular components in swine mammary secretion

Cellular components in swine mammary secretions were first described by Evans et al. in 1982. Colostrum and milk were collected from multiparous, Large White sows (n = 3) following intravenous injection with oxytocin to stimulate milk letdown. Cells harvested from the samples were counted in a Neubauer hemocytometer and cells viability assessed using a trypan blue exclusion procedure. Total cell yield was highest in colostrum (1×10^7 cells per ml) and then decreased by day post-partum. The population of cells in colostrum

consisted of 71.7% neutrophils, 26.4% lymphocytes, 1.3% macrophages, 0.4% epithelial cells, and 0.2% eosinophils. The number and proportion of each cell type in mammary secretions varied by day post-partum, but tended to decrease until 20 days post-partum. Later estimates of the number and proportion of cell types in mammary secretions vary widely (Evans et al., 1982; Hurley and Grieve, 1988; Magnusson et al., 1991; Magnusson, 1999; Lee et al., 1983; Schollenberger et al., 1986; Wuryastuti et al., 1993). Magnusson et al (1991) suggested that this variation in cell numbers and types reflects both the developmental stage of the mammary gland and the physiological and/or immunological conditions of the sow.

Lymphocytes

Le Jan et al. (1994) evaluated cells in sow milk and found that 10 to 25% were lymphocytes, among which 70 to 90% were CD4+ and CD8+ T lymphocytes. In colostrum, Le Jan et al. (1994) reported 57% as CD4+ or CD8+ presenting cells. Notably, this work did not identify dual CD4-/CD8- T lymphocytes, which will have led to inaccuracies in the reported proportions of CD4+ and CD8+ T lymphocytes.

T lymphocytes in mammary secretions are a select population of cells that migrated from circulation (Le Jan, 1996). CD4+ or CD8+ T lymphocytes in mammary secretions are known to differ from circulating T lymphocytes in specific responses, e.g., they proliferate in response to exposure to ovalbumin (Evans et al., 1982), and do not express interleukin-2 (IL-2) receptors, except when stimulated with mitogenic substances (Le Jan et al., 1994). Large questions remain, however. For example, characteristics and functions of γ-δ T lymphocytes in swine mammary secretions are largely under-researched.

Research in humans suggest that B lymphocytes in mammary secretions may have been plasmablast and/or plasma cells that were selectively transported across epithelial cells of the mammary gland alveoli (Bush and Beer, 1979). Like T lymphocytes, B lymphocytes in mammary secretions differ from their counterparts in the circulatory system. Only 2% of B lymphocytes in mammary secretions contain complement receptors versus 31% of B lymphocytes in circulation (Schollenberger et al., 1986). Overall, the function of B

lymphocytes in mammary secretions and their contribution to passive immunity in the piglet is unclear.

Phagocytic cells

Phagocytic cells in swine mammary secretion include both neutrophils and macrophages, with neutrophils constituting the largest proportion, i.e. 72% in colostrum and 32 to 55% in milk (Evans et al., 1982). Under light and electron microscopy, Lee et al. (1983) found that neutrophils and macrophages recovered from mammary secretions had phagocytic vacuoles containing lipid, casein, and cellular debris.

Schellenberger et al. (1986) confirmed that neutrophils and macrophages in sow mammary secretion were capable of phagocytosis, but found they were not capable of inactivating *E. coli*. Schellenberger et al. (1986) estimated the killing index of phagocytic cells in circulation at between 0.14 and 0.25, i.e., they effectively inactivated phagocytized *E. coli*. In contrast, the intracellular killing index of neutrophils and macrophages in sow mammary secretion was > 1, i.e., phagocytized *E. coli* multiplied because the cells were unable to kill them. Evans et al. (1982) compared neutrophils and macrophages in sow mammary secretion versus alveolar macrophages and blood neutrophils in terms of their ability to phagocytosis yeast cells. The intracellular killing index of mammary secretion neutrophils was not significantly different than alveolar macrophages and blood neutrophils, but the activity of mammary secretion macrophages was significantly lower than the other phagocytic cells ($p < 0.1$).

Cumulatively, these studies suggested that phagocytic cells in swine mammary secretion are capable of phagocytosis, but may lack some intracellular functions. This reports are consistent with reports that neutrophils recovered from human mammary secretions showed a reduction in functionality, e.g., motility, phagocytosis, respiratory burst, and polymorphonuclear (PMN)-mediated cytotoxicity, compared to serum-derived cells (Kohl et al., 1980; Nair et al., 1985; Ozkaragoz et al., 1988; Schollenberger et al., 1986).

Epithelial cells

In swine, mammary alveolar epithelial cells serve an important role in transferring antibody from the parenchyma into the alveolae (Hurley and Theil, 2011), as will be fully described in the next section. In other respects, our understanding of the function of epithelial cells in swine mammary secretions and/or their role in neonatal immunity is incomplete and primarily descriptive. In mammary secretions, sloughed epithelial cells constitute ~2.5% of cells in colostrum and 35 to 80% of cells in milk (Boutinaud and Jammes, 2002). Lee et al. (1983) reported that the number of epithelial cells increased from the day one through day 28 of lactation. The phenotype, composition, and perhaps the functionality of epithelial cells change over the course of lactation. When comparing epithelial cells in colostrum to those in milk, Le Jan et al., (1993) found that colostral cells were smaller (9-15 μm vs 15-40 μm), expressed less cytoplasmic secretory component and no membrane secretory component, contained little or no intracellular IgA, and were unable to replicate under *in vitro* conditions. Just 10% of the epithelial cells in colostrum versus 66% of the epithelial cells in milk contained secretory component (J chain) and intracytoplasmic dimeric IgA. Le Jan (1996) hypothesized that, similar to epithelial cells in the mammary secretions of other species, swine mammary secretion epithelial cells might be able to produce cytokines and function as antigen-presenting cells. For example, epithelial cells in bovine mammary secretion secrete some innate immune factors, including lactoferrin, β-defensin, and lipopolysaccharide binding protein (Miles, 2004; Sanchez et al., 1992; Swanson et al., 2004). Milk fat globule secreted by epithelial cells in bovine mammary secretions contains membrane components with antimicrobial activity, e.g., xanthine oxidase and sphingolipids (Hancock et al., 2002; Sprong et al., 2001).

Antibodies in swine mammary secretions

Antibody isotypes were first identified in human plasma in 1937 (Tiselius) and milk in 1962 (Hanson and Berggård). Thereafter, Porter (1969) identified antibody isotypes in swine milk as 79.7% IgG, 14.1% IgA, and 6.2% IgM. Curtis and Bourne (1971) reported that the immunoglobulin composition of swine mammary secretions differed between colostrum and milk. Colostrum contained an average of 61.8 mg per ml IgG1, 40.3 mg per ml IgG2, 9.7 mg per ml IgA, and 3.2 mg per ml IgM. In contrast, milk (at 72 h of lactation) contained an

average of 1.9 mg per ml IgG, 1.3 mg per ml IgG2, 3.4 mg per ml IgA, and 1.2 mg per ml of IgM.

Bourne and Curtis (1972) identified the sources of IgG, IgA, and IgM in sow mammary secretion by injecting sows (n = 18) intravenously with ^{125}iodine-labelled IgG, IgA, and IgM 7 days prior to farrowing and then collecting mammary secretion samples and measuring the proportion of serum-derived immunoglobulin. In colostrum, 100% of IgG, 24 to 54% of IgA, and 70 to 100% of IgM came from serum. In contrast, 20 to 38% of IgG, 12 to 27% of IgA, and 4 to 32% of IgM in milk came from serum. By default, the remainder of immunoglobulins are produced locally, i.e., within the mammary parenchyma.

Thus, antibody in mammary secretions is derived from two distinct sources: serum antibody produced by plasma cells in lymphoid follicles reaches the mammary glands via the circulatory system and antibody (IgG, S-IgA, S-IgM) produced by plasma cells located in the mammary tissue (Brantzaeg, 1981). Mammary tissue plasma cells originate as B lymphocytes that have been stimulated by exposure to antigen. These cells mature to plasma cells in mucosal lymphoid follicles, then re-enter the circulatory system and migrate to the mammary parenchyma. The migrating cells bind to "mucosal addressin cell-adhesion molecule 1" (MADCAM1) presented by cells in the mammary parenchyma via adhesive molecule-like α4, β7-integrin on the plasma cell membrane (Brandtzaeg, 2010).

Transport of antibody into mammary secretions

Regardless of source, immunoglobulins in mammary gland tissue are transcytosed into the mammary gland alveolae via antibody isotype-dependent mechanisms, one for IgG and one for secretory IgA (S-IgA) and IgM (S-IgM).

IgG transport is mediated by the binding of IgG heavy chain and the neonatal Fc receptor (FcRn) on the basolateral surface of the mammary epithelial cells. Specifically, the Fc portion of IgG binds with high affinity to FcRn at pH <6.5. The receptor-bound IgG is then endocytosed and transported to the mammary gland alveolae (apical end of the epithelial cell). At the physiological pH of mammary secretions (7.2 to 7.8), the FcRn receptor loses

its affinity for IgG and releases the molecule into the mammary alveolae (Roopenian and Akilesh, 2007).

The selective transport of IgG from serum results in the concentration of IgG in mammary secretions during late pregnancy; a period that coincides with high progesterone and low estrogen levels (Butler, 1998; Chamley et al., 1973; Hammer and Mossmann, 1978; Killian et al., 1973; Porter, 1988; Schnulle and Hurley, 2003; Watson, 1980). On the day of parturition, the concentration of IgG in colostrum is three times that of the sow's serum. For this reason, piglet serum IgG levels during the colostral period are often higher than those found in the dam's serum (Newby et al., 1982; Porter, 1988; Simpson-Morgan and Smeaton, 1972). Likewise, this explains why sows seronegative for certain pathogens may have neonates that are seropositive for the same pathogen.

The transepithelial transport of S-IgA and S-IgM is mediated by the polymeric immunoglobulin receptor (pIgR) on mammary epithelial cells. pIgR binds the J-chain peptide of polymeric immunoglobulins (dimeric IgA and pentameric IgM), then the pIgR-bound polymeric immunoglobulin is internalized by endocytosis and transported from the basolateral surface to the apical surface of the epithelial cells. At the apical surface, the pIgR is cleaved by proteolytic enzymes and the antibody is released into the mammary alveolae. The pIgR secretory component remains bound to the immunoglobulin J-chain (Hurley and Thiel, 2011; Otten and Egmond, 2004). pIgR mediates the transport of both IgM and IgA in mammary secretions, for which reason the free secretory component can be detected in both colostrum and milk (Kumura et al., 2000; Le Jan, 1993).

In particular, the transport of S-IgA, and therefore its concentration in mammary secretions, mirrors the expression of pIgR during lactation (De Groot et al., 2000). Although the hormonal regulation of the expression of pIgR on sow mammary epithelial cells has not been described, *in vitro* work by Rosato et al. (1995) using mammary tissue explants from pregnant or lactating rabbits suggested that the expression of pIgR was stimulated by an increase in prolactin and a decline in progesterone and estradiol concentrations during lactation.

Acquisition of maternal immunity by the neonatal piglet

The immature systemic and mucosal adaptive immune systems of newborn piglets are characterized by limited peripheral lymphoid cells, undeveloped lymphoid tissues, and the absence of effector and memory T-lymphocytes (reviewed by Sinkora and Butler, 2009). To partially fill this lack, lymphocytes ingested by suckling piglets are able to cross the duodenal and jejunal epithelium by intercellular migration for at least one week after birth (Bianchi et al., 1999; Tuboly et al., 1988; Williams, 1993). For reasons that are not yet clear, only maternally-derived cells are able to cross (Tuboly et al., 1988; Williams, 1993), i.e., cross-fostered piglets cannot absorb cells from a substitute dam (Bandrick et al., 2011). Thereafter, these cells migrate to mesenteric lymph nodes and a variety of other tissues, including liver, lung, spleen, and the lamina propria and submucosal spaces of the duodenum and jejunum (Tuboly et al., 1988; Tuboly and Bernath, 2002; Williams, 1993). Williams (1993) demonstrated that colostral mononuclear leukocytes could be detected in liver, lung, spleen, and mesenteric lymph nodes 24 to 48 hours after suckling.

These cells retain functionality within the neonate. Maternally-derived immune cells from human babies displayed weak cytotoxicity against Herpes simplex virus-infected target cells (Kohl et al., 1980). In piglets that received colostral cells, peripheral blood mononuclear T-lymphocytes showed a significantly higher response to mitogens, e.g., phytohemagglutinin and concavalin A, compared to controls and peripheral B-lymphocytes showed significant responses to pokeweed mitogen (Williams, 1993). In an investigation of one week-old piglets, Bianchi et al. (1999) postulated that IgG secretory cells detected in spleen, peripheral lymph nodes, and bone marrow were the progeny of B cells originally provided in colostrum. There is limited published research on the role of colostral cells in protecting piglets against pathogens. However, it has been shown that the transfer of peripheral blood mononuclear cells from adult pigs to neonates delayed the onset of transmissible gastroenteritis virus (TGEV) (Cepica and Derbyshire, 1984).

Piglets are born agammaglobulinemic and do not become fully immunologically competent until about four weeks of age (Blecha, 2001; Langel et al., 2016; Salmon et al., 2009).

Transmission of swine maternal antibody to neonates was first demonstrated by Speer et al., (1959). Sows intravenously immunized with *E. coli* were shown to produce mammary secretions with antibodies against the pathogen. Thereafter, colostrum collected from the immunized sows was fed to piglets born to *E. coli*-naïve sows. Testing of blood samples from the piglets showed that *E.coli*-specific antibody was detectable in piglet serum within 12 h after colostrum administration.

Until they become immunocompetent, the antibodies provided in colostrum and milk play a crucial role in the protection of the piglets against infectious disease. Ingested maternal immunoglobins remain intact within the neonatal digestive tract because the proteolytic activity in the digestive tract is low and is further reduced by "sow colostrum trypsin inhibitor (SCTI)" (Jensen, 1978). Trypsin is able to cleave immunoglobulins, but SCTI irreversibly binds trypsin, thereby protecting proteins in mammary secretions (Chamberlain et al., 1965; Kunitz, 1947).

Transcytosis of maternal immunoglobulins across neonatal intestinal epithelial cells is initiated by one of two mechanisms: non-selective endocytosis or specific receptor-mediated binding of antibody (Bush and Staley, 1980; Harvey and Lecce, 1979; Pácha, 2000). Non-selective endocytosis relies on the fact that the neonatal small intestine is lined with mucosal epithelial cells temporarily able to absorb intact macromolecules (Hurley and Thiel, 2011).

Immunoglobulins that transcytose the enterocytes are released in the lamina propria, diffuse to the intestinal lymphatic system, and enter the circulatory system (Moog, 1979; Murata and Namioka, 1977). Colostral IgG, a reflection of the sow's antibody profile, appears in the neonatal circulatory system within 48 hours after birth (Porter, 1988; Watson, 1980; Wilson et al., 1989). In addition, absorbed maternal IgM, IgA, and IgG can undergo transudation through the epithelial cells to the intestinal mucosal surfaces of the piglet where it plays a major role in protecting neonates against enteric pathogens, e.g., porcine epidemic diarrhea virus (PEDV), TGEV and rotavirus (Bohl et al., 1972a; Bohl et al., 1972b; Bradley et al., 1990; Poonsuk et al., 2016a; Poonsuk et al., 2016b; Saif et al., 1972; Saif, 1999; Ward et al., 1996).

This neonate's ability to absorb maternal antibody exists for a very limited period of time. According to Murata and Namioka (1977), pinocytosis of colostral immunoglobulins disappeared by 2 hours after birth in the duodenum, 48 hours in jejunum, and 72 hours in the ileum ("gut closure"). In addition, the mucosal epithelium is completely replaced by intestinal epithelial cells incapable of transcytosis within 9 days after birth (Moog, 1979; Smith and Jarvis, 1978). Although these "new" cells can pinocytose macromolecules, they cannot complete the transfer of immunoglobulins to the lamina propria (Pácha, 2000; Rooke and Bland, 2002).

After gut closure occurs, the majority of macromolecules in the intestinal tract are degraded by digestive enzymes, but S-IgA and S-IgM are stabilized by binding to secretory component (Brandtzaeg, 1981). Some of these intact antibodies are transcytosed via antibody-specific FcRn and pIgR receptors on intestinal epithelial cells (Pácha, 2000; Stirling et al., 2005); a process stimulated by epithelial growth factor (EGF) and transforming growth factor β (TGF-β) in colostrum (Danielsen et al., 2006; Jensen et al., 2001).

Maternal immunity and swine health: past, present, and future applications

The timeline of scientific discoveries that led to the recognition of maternal immunity was reviewed in Section 2.0. Directly pertaining to swine, Friedberger and Frohner (1908) were the first to document the role of maternal antibody in piglet health, i.e., piglet susceptibility to erysipelas was inversely related to the level of maternal immunity. Subsequently, McArthur (1919) found that 70% of one-day-old piglets born from "hog cholera" (classical swine fever virus, CSFV) immune sows were resistant to CSFV infection.

Overall, however, the relevance of passive immunity to piglet health was slow to gain recognition in swine medicine. Despite the seminal work on classical swine fever virus (De Schweinitz and Dorset, 1903; Dorset et al., 1904) and the creation of a substantial body of work on bacterial infections in swine (see Merchant, 1940), little work was done on porcine maternal immunity until after World War II (see Dunne, 1958). After WWII, the role of

maternal immunity in neonatal protection was demonstrated against a wide range of pathogens of swine (Table 1). As management and production practices improved over this period, the natural question was, *How can breeding herd immunity be manipulated so as to optimize maternal immunity in neonates?*

There are essentially two methods for managing immunity in the host: intentional exposure to the agent or vaccination. Intentional exposure is based on choosing the time, place, and route by which the host will be exposed to material presumed to be contaminated with the agent. This approach was used in smallpox (variola virus) control as early as 1000 A.D. (Parrino et al., 2006). "Feedback", infecting sows by exposing them to biological material (tissues, manure) contaminated with the pathogen of interest is a form of intentional exposure that has been widely used in swine production. A search of the literature suggested that Bay et al. (1953) was the first to document this process. Using an inoculum prepared from macerated gastrointestinal tracts recovered from TGEV-infected pigs, pregnant sows were exposed orally (n = 10), intramuscularly (n = 4), subcutaneously (n = 2), or served as uninoculated controls (n = 10). The piglets (n = 200) from these sows were then orally exposed to the same inoculum at 3 to 7 days of age. Piglet survivability by group was 50 of 70 (71%) from orally exposed sows, 14 of 27 (52%) from intramuscularly exposed sows, 0 of 23 from subcutaneously exposed sows, and 12 of 80 (15%) from uninoculated control sows. Feedback has significant drawbacks, including non-uniform and uncontrolled pathogen exposure, the unintended transmission of adventitious agents likewise present in the inoculum, and the perpetuation of virulent viruses in the herd (Clement et al., 2016; Chase and Lunney, 2012; Saif et al., 2012).

Based on earlier work in rinderpest (Edington, 1897), USDA scientists developed a method to control hog cholera based on intentional exposure of swine to CSFV and simultaneous administration of anti-CSFV antibody (Dorset et al., 1908). Abuse of this method, i.e., preparation and sales of inadequate or substandard products by commercial entities, led to the Virus-Serum-Toxin Act of 1913 and the establishment of specific criteria for the production of vaccines (Conner, 1952; Skidmore, 1927). Even so, the simultaneous method continued in

use until effective killed (McBryde and Cole, 1936) and live-attenuated CSFV vaccines (Koprowski et al., 1946; Baker, 1946) became available.

The advent of vaccines, particularly those produced under the guidelines of the Virus-Serum-Toxin Act of 1913, addressed many of the pitfalls of intentional exposure by providing assurance that the inoculum was pure, safe, potent, and efficacious. Vaccination of sow herds has been used to stimulate maternal immunity and protect piglets against various pathogens, e.g. porcine parvovirus (Antonis et al., 2006), porcine circoviruses (Chae, 2012), porcine reproductive and respiratory syndrome virus (Meng, 2000), pseudorabies (Aujeszky's disease) virus (Mettenleiter et al., 1994), *Leptospira spp.* (Fish and Kingscote, 1973), and *Mycoplasma hyopneumoniae* (Ruiz et al., 2003). In general, vaccines that stimulate immunity in the dam will improve maternal immunity in the neonate, but there are exceptions. Bohl et al. (1972b) showed that the route of immunization affected the antibody isotypes present in mammary secretions. In particular, sows orally inoculated or naturally infected with virulent TGEV developed higher levels of neutralizing antibodies and higher S-IgA responses in mammary secretions than sows intramuscularly injected with TGEV. In particular, piglets nursing orally inoculated or naturally infected sows received higher levels of TGEV-specific IgA and survived exposure to TGEV at a higher rate. In 1975, Bohl and Saif discovered that TGEV-specific IgA in milk reflected "productive" intestinal replication of the virus, whereas parenteral antigenic exposure stimulated TGEV-specific IgG. As reviewed in Section 5.2, enteric infections stimulate plasmacytes to mature into plasma cells which then migrate to the mammary parenchyma where they produce the IgA that is incorporated into mammary secretions. For these reasons and until vaccines capable of stimulating pathogen specific S-IgA are available, feedback will be needed to provide adequate levels of maternal immunity against certain enteric pathogens in piglets, e.g. TGEV and PEDV.

These concepts are clear and well-established: Maternal immunity is transferred from sows to their piglets via mammary secretions. This process provides protection against infectious agents during the lactation period and until the immune system of the piglet is more fully developed. Maternal immunity can be manipulated by intentional exposure and/or

vaccination to enhance herd health and productivity. The challenge to implementing and optimizing this approach in the field is that quantitative methods for measuring lactogenic immunity have not been fully developed and, in many cases, the levels of maternal immunity required to protect piglets against specific agents are not well-characterized. The issue is compounded by the fact that pre-farrow sow serum antibody levels do not directly reflect the antibody levels she will provide in colostrum and/or milk. Antibodies can be directly measured in colostrum and/or milk, but at that point it is too late to stimulate maternal immunity. Therefore, methods to measure immunity in gestating animals and predict the level of lactogenic immunity they will provide their piglets is an area in need of investigation.

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Table

Table 1. Pathogens against which maternal immunity can provide protection to piglets

Viral Pathogens	References
Classical swine fever virus	Vandeputte et al., 2001
Foot-and-mouth disease virus	Salt et al., 1998; Kitching and Salt, 1995
Influenza virus	Kitikoon et al., 2006
Japanese encephalitis virus	Scherer et al., 1959
Porcine bocavirus	Zhai et al., 2010
Porcine circovirus	Harms et al., 2001; McKeown et al., 2005
Porcine epidemic diarrhea virus	Poonsuk et al., 2016a, b; Jung and Saif, 2015
Porcine parvovirus	Damm et al., 2002
Porcine reproductive and respiratory syndrome virus	Chung et al., 1997
Porcine respiratory coronavirus	Sestak et al., 1996
Porcine rotavirus	Askaa et al., 1983; Ward et al., 1996
Pseudorabies (Aujeszky's disease) virus	Tielen et al., 1981; Weigel et al., 1995
Transmissible gastroenteritis virus	Saif and Sestak 2006
Vesicular stomatitis virus	Hanson, 1958
Bacterial Pathogens	**References**
Actinobacillus pleuropneumoniae	Vaillancourt et al., 1988; Vigre et al., 2003
Actinobacillus suis	Lapointe et al., 2001
Bordetella bronchiseptica	Elahi et al., 2006; Haesebrouck et al., 2004
Brachyspira hyodysenteriae	Diego et al., 1995
Brucella suis	Manthei et al. 1952; Manthei, 1958
Erysipelothrix rhusiopathiae	Opriessnig et al., 2004; Wood, 1999
Escherichia coli	Isaacson et al., 1980
Haemophilus parasuis	Blanco et al., 2004; Oliveira et al., 2003; 2004
Lawsonia intracellularis	Riber et al., 2015
Leptospira interrogans	Millar et al., 1987
Mycoplasma hyopneumoniae	Morris et al., 1994
Pasteurella multocida	Maes et al., 1996
Salmonella choleraesuis	Wilcock, 1979
Streptococcus suis	Torremorell et al., 1997
Parasitic Pathogens	**References**
Ascaris suum	Jungersen, 2002
Cryptosporidium spp.	Zintl et al., 2007
Hyostrongylus rubidus	Smith and Herbert, 1975
Isospora suis	Lindsay et al., 1999; Wieler et al., 2001
Toxoplasma gondii	Opsteegh et al., 2011
Trichinella spiralis	Berends et al., 2011
Trichuris suis	Nejsum et al., 2009

CHAPTER 3. DOES CIRCULATING ANTIBODY PLAY A ROLE IN THE PROTECTION OF PIGLETS AGAINST PORCINE EPIDEMIC DIARRHEA VIRUS?

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Modified from a manuscript published in PLoS ONE (2016) 11(4):e0153041

Abstract

The contribution of circulating antibody to the protection of naïve piglets against porcine epidemic diarrhea virus (PEDV) was evaluated using a passive antibody transfer model. Piglets (n = 62) derived from 6 sows were assigned to one of 6 different treatments using a randomized block design which provided for allocation of all treatments to all sows' litters. Each treatment was designed to achieve a different level of circulating anti-PEDV antibody via intraperitoneally administration of concentrated serum antibody. Piglets were orally inoculated with PEDV (USA/IN/2013/19338E, 1 x 10^3 TCID50 per piglet) 24 hours later and then monitored for 14 days. Piglets remained with their dam throughout the experiment. Sow milk samples, piglet fecal samples, and data on piglet clinical signs, body weight, and body temperature were collected daily. Fecal samples were tested by PEDV real-time reverse transcriptase PCR. Serum, colostrum, and milk were tested for PEDV IgG, IgA, and virus-neutralizing antibody. The data were evaluated for the effects of systemic PEDV antibody levels on growth, body temperature, fecal shedding, survival, and antibody response. The analysis showed that circulating antibody partially ameliorated the effect of PEDV infection. Specifically, antibody-positive groups returned to normal body temperature

faster and demonstrated a higher rate of survivability than piglets without PEDV antibody. When combined with previous literature on PEDV, it can be concluded that both maternally-derived IgG in colostrum and maternal secretory IgA in milk contribute to the protection of the neonatal pig against PEDV infections.

Introduction

The *Coronaviridae* is a large and complex family of enveloped, single-stranded, positive-sense RNA viruses that cause enteric and respiratory disease in humans and animals. Recently-emerged coronaviruses include the severe acute respiratory syndrome (SARS) virus that caused outbreaks of respiratory disease in humans in 2002-2003 and the Middle East respiratory syndrome (MERS) virus identified in 2012 (Zaki et al., 2012). Contemporary work suggests that bat and bird species are the natural reservoirs of coronaviruses (Muradrasoli et al., 2010).

Five coronaviruses are recognized in swine: three alphacoronaviruses (transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), and porcine epidemic diarrhea virus (PEDV)), one betacoronavirus (porcine hemagglutinating encephalomyelitis virus (PHEV)), and one species of porcine deltacoronavirus (PDCoV) (Doyle and Hutchings, 1946; Pensaert et al., 1986; Chasey and Cartwright, 1978; Greig et al., 1962; Wang et al., 2014; Werdin et al., 1976). PEDV, TGEV and PDCoV primarily cause enteric infections in pigs. PRCV is the result of deletion and mutation of the spike gene of TGEV. This virus has a predilection for the respiratory tract, but also has the capacity to produce enteric disease (Wesley et al., 1990). In contrast, PHEV infection ("vomiting and wasting disease") produces encephalomyelitis, rather than enteritis, and thus is not often considered when differentiating enteric infections (Greig et al., 1962).

Among the porcine coronaviruses, PEDV has received considerable attention because recently emerged highly virulent strains have caused significant morbidity and mortality in neonatal pigs (Chen et al., 2014). Catastrophic outbreaks of PEDV were reported in China, Thailand, and Korea beginning in 2007 (Song and Park, 2012). Following its detection in the

U.S. in April 2013 (Stevenson et al., 2013), PEDV is estimated to have caused the deaths of 8 million piglets and economic losses of $481 to $929 million (USD) in 2014 (Paarlberg, 2014).

The primary site of PEDV replication is the cytoplasm of villous enterocytes throughout the small intestine. Infection causes epithelial cell degeneration and villous atrophy, which leads to diarrhea, dehydration, and prolonged shedding of PEDV in feces (Jung et al., 2014; Madson et al., 2014). PEDV viremia has also been reported during the acute stage of infection in young pigs (Jung and Saif, 2015; Jung et al., 2014; Madson et al. 2015; Opriessnig et al., 2014). The most common clinical consequence of PEDV infection is diarrhea, i.e. watery and flocculent feces, often accompanied by vomiting (Saif et al., 2012). Morbidity and mortality is highly age-dependent, with neonatal pigs the most severely affected. Thus, an outbreak in a naïve swine population may result in 90% mortality in piglets ≤ 2 weeks of age and ≤ 40% mortality in 2- to 4-week-old pigs (Stevenson et al., 2013). This age-dependent variation in mortality is likely the result of slower villus-epithelial repopulation and less developed immune systems in neonatal pigs (Annamalai et al., 2015; Jung and Saif, 2015; Moon, 1971; Madson et al., 2014; Shibata et al., 2000). Experimentally-infected 3-week-old pigs showed a significant reduction in average daily gain during the first week post-inoculation and no compensatory weight gain in the following 4 weeks (Madson et al., 2014). In the field, Olanratmanee et al. (2010) reported that PEDV infection in pregnant gilts and sows may also have contributed to reduced reproductive performance, including a 12.6% decrease in farrowing rate, a 5.7% increase in the return rate, a 1.3% increase in the abortion rate, and a 2.0% increase in the number of mummified fetuses per litter.

It is generally accepted that lactogenic immunity, i.e., anti-PEDV secretory IgA in milk, is central to limiting the replication of PEDV in the intestinal tract and protecting piglets against clinical disease (Pensaert, 1999). This concept is primarily derived from research showing that sows with higher anti-TGEV SIgA levels in milk were better able to protect their piglets against clinical TGE (Bohl et al., 1972 a,b; Bohl and Saif, 1975; Saif et al., 2012). These observations are the foundation upon which successful TGEV prevention and

control strategies have been based for over 50 years (Doyle, 1958). However, dissimilarities between immunity to PEDV versus immunity to TGEV have not been closely examined and deserve investigation. The question addressed in this project was the effect of colostral (passive) antibody on the protection of neonates against PEDV. Specifically, the objective of this experiment was to quantify the impact of circulating anti-PEDV antibody on the course of PEDV replication and clinical disease in the neonatal pig using a passive transfer model.

Materials and Methods

Experimental design

The study was conducted under the approval of the Iowa State University Office for Responsible Research (ISU #2-14-7736-S). Piglets (n = 62) from 6 PEDV indirect fluorescent antibody (IFA)-negative sows were intraperitoneally (IP) administered concentrated serum antibody sufficient to achieve one of 6 targeted levels of circulating anti-PEDV antibody. All piglets were inoculated with PEDV 24 h later and were then observed daily until day post inoculation (DPI) 14 or until humane euthanasia was necessary. Each day, sow milk and piglet fecal samples and data on piglet clinical signs, body weight, and body temperature were collected. Serum samples were collected from sows at DPIs -7 and 14 and from piglets at DPIs -1, 0, and 14 or at the time of humane euthanasia. Fecal samples were tested by PEDV real-time reverse transcriptase PCR (rRT-PCR). Serum, colostrum, and milk were tested for PEDV IgG, IgA, and virus-neutralizing antibody. The data were evaluated for the effects of systemic PEDV antibody levels on the outcomes measured.

Porcine epidemic diarrhea virus (PEDV) inoculum

The PEDV isolate used in the study (USA/IN/2013/19338E), was isolated in 2013 at the Iowa State University Veterinary Diagnostic Laboratory from piglet small intestine submitted from an Indiana swine farm (Chen et al., 2014). The inoculum used in this study was the 7th passage of the virus on cell culture.

For use in this study, the virus was serially propagated on Vero cells (African green monkey kidney) in flasks using methods described elsewhere (Hofmann and Wyler, 1988; Chen et al.,

2014). In brief, Vero cells (ATCC® CCL-81™, American type culture collection, Manassas, VA) were cultured in 25 cm^2 flasks (Corning®, Corning, NY) using maintenance medium (minimum essential medium (MEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 0.05 mg per ml gentamicin (Life Technologies), 10 units/ml penicillin (Life Technologies), 10 µg per ml streptomycin (Sigma-Aldrich) and 0.25 µg/ml amphotericin (Sigma-Aldrich). Maintenance medium was decanted from contiguous cell monolayers, the monolayer was washed twice with maintenance medium, and then the flask was inoculated with 0.5 ml of a mixture of PEDV and post-inoculation medium (MEM supplemented with tryptose phosphate broth (0.3%) (Sigma-Aldrich), yeast extract (0.02%) (Sigma-Aldrich) and Trypsin 250 (5 µg/ml) (Sigma-Aldrich). Flasks were then incubated at 37°C with 5% CO_2 for 2 h to allow virus adsorption after which 5 ml of post-inoculation medium was added to each flask without removing viral inoculum. Flasks were incubated at 37°C with 5% CO_2 until cytopathic effect (CPE) was observed and then subjected to one freeze-thaw cycle (-80°C). The contents were harvested, centrifuged at 3,000 x *g* for 10 min at 4°C to remove cell debris, aliquoted into 2.0 ml microcentrifuge tubes, and stored in -80°C until used.

PEDV titration was performed on confluent Vero cells monolayers grown in 96-well plates (CoStar™, Corning®). Eight 10-fold dilutions of virus stock solution were made using post-inoculation medium. Five wells were inoculated with 100 µl at each dilution, plates were incubated at 37°C with 5% CO_2 for 1 h, and then 100 µl post-inoculation medium was added. Plates were incubated at 37°C with 5% CO_2 for 5 days, after which wells were subjected to IFA staining and evaluated for the presence of virus. Wells with specific staining were classified PEDV-positive. Based on the titration results, the 50% endpoint was calculated as 1×10^5 $TCID_{50}$/ml using the Reed-Muench method (Reed and Muench, 1938).

Animals and animal care

The experiment was conducted in the Iowa State University Livestock Infectious Disease Isolation Facility (LIDIF), a biosafety level 2 research facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The facility was equipped with a single-pass non-recirculating ventilation system that provides directional

flow from low contamination areas to high contamination areas and zones of negative pressure to prevent control airborne contamination from area-to-area or room-to-room. Each room was ventilated separately and humidity and temperature was strictly controlled.

Seven clinically healthy pregnant sows were acquired from one commercial sow farm at day 110 of their second gestation. To verify their negative status, sow fecal swabs were tested for PEDV, TGEV, and PDCoV using agent-specific rRT-PCRs and serum samples were tested for PEDV antibody using IFA.

Sows were housed in Danish-style free stall farrowing crates (Thorp Equipment Inc., Thorp, WI) and supplemental heat was provided for piglets. Animals were closely observed from the time they entered LIDIF to the end of the observation period by researchers, animal caretakers, and veterinary staff. All sows had been bred on the same day. To induce parturition, all sows were administered 10 mg of dinoprost tromethamine (Lutalyse®, Zoetis Inc., Florham Park, NJ) 24 h prior to the expected farrowing date, i.e., day 113 of gestation. Sows completed farrowing either 1 (n = 1), 2 (n = 5), or 4 (n = 1) days after induction. All viable piglets (n = 74) were ear-tagged and administered 1 ml iron hydrogenated dextran (VetOne®, Boise, ID) and 5 mg (0.1 ml) ceftiofur sodium (Excenel®, Zoetis). Piglets remained with their dam continuously throughout the 2-week observation period.

Implementation of the experiment

Concentrated PEDV antibody

The procedure used to precipitate swine serum proteins and antibodies was a modification based on previous publications (Hebert et al., 1973; Grodzki and Berenstein, 2010). Specifically, swine serum proteins and serum antibody were precipitated by single fractional precipitation using 30% and then 40% ammonium sulfate, respectively. The entire process was conducted in an environmental chamber (Caron®, Marietta, OH) maintained at 4°C. Initially, whole blood was collected from two humanely euthanized and exsanguinated PEDV antibody-positive sows (ISU #2-14-7736-S). Serum was harvested and stored in 1 L bottles (Biotainer™, Nalge Nunc Corp., Rochester, NY) at -20°C. For the first precipitation, the serum was thawed for 24 h at 4°C, the volume of saturated ammonium sulfate (Sigma-

Aldrich) calculated to achieve 30% concentration was added in a drop-by-drop fashion while stirring continuously, and then the mixture was incubated at 4°C for 16 h with continued stirring. Following incubation, the mixture was centrifuged (4°C) at 4,000 x *g* for 10 min to remove protein aggregates and less soluble proteins. For the second precipitation, the volume of the supernatant (antibody fraction) was measured, additional saturated ammonium sulfate was added as before to achieve a final concentration of 40%, and then the mixture was incubated for 16 h at 4°C with stirring. Thereafter, the mixture was centrifuged (4°C) at 4,000 x *g* for 10 min to recover the antibody fraction. The pelleted antibody was gently resuspended with PBS (1X pH 7.4) at a 1:5 (pellet:PBS) volume ratio. To remove salts, the solution was dialyzed in 250 ml dialysis flasks (Pierce®, Thermo-Fisher Scientific, Waltham, CA) floating vertically in ~15 liters of 4°C, continuously stirred PBS (1X pH 7.4). The entire volume of PBS was replaced every 4 h for 5 times and then the antibody solution was concentrated by polyacrylamide gel dialysis (Spectra/Gel® Absorbent, Spectrum Laboratories, Inc. Rancho Dominguez, CA). The concentrated antibody solution was then aliquoted into 50 ml centrifuge tubes and stored at -80°C until used.

Treatments

The concentrated PEDV antibody solution was thawed at 22°C for 2 h and then 2-fold diluted with PBS (1X pH 7.4) to create 6 treatments, i.e., 5 dilutions (1:80; 1:160; 1:320; 1:640; 1:1280) of the antibody solution plus an antibody-negative control (PBS 1X pH 7.4). To assign treatments (Table 1), piglets were blocked by sow and randomized to treatments in a randomized block design using statistical software R (R 3.2.0, R foundation®). Notably, all piglets remained with their dam, but all treatments were represented within each litter. Treatments were administered by intraperitoneal inoculation at the rate of 1.35 ml of the solution per kg of piglet bodyweight.

PEDV inoculation

A virulent U.S. PEDV isolate (USA/IN19338/2013) was used as the challenge virus in this study. Isolation, propagation and characterization of this isolate was previously described (Chen et al, 2014). On DPI 0, the PEDV stock solution (passage 7 in cell culture, 1 x 10^5 $TCID_{50}$/ml) was diluted to an estimated concentration of 1 x 10^3 $TCID_{50}$/ml, mixed 1:4 with

milk replacer (Esbilac®, PetAg Inc., Hampshire, IL) and administered orally (5 ml) to each piglet. Thereafter, sows were monitored daily for diarrhea, milking ability, anorexia, and alertness. Piglets were monitored daily for diarrhea, rectal body temperature, dehydration, and ability to stand, walk, and suckle. Animals unable to suckle, reluctant to stand, or demonstrating ≥ 10% dehydration based on skin tenting were euthanized by intravenous administration of pentobarbital sodium (Fatal-Plus®, Vortech Pharmaceuticals, MI) at a dose of 100 mg/kg.

Biological sample collection

Serum

Serum samples for antibody testing were collected from sows (DPIs -7, 14) and piglets (DPIs -4, 0, 14). Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection system (Becton Dickson, Franklin Lakes, NJ) and serum separation tubes (Kendall, Mansfield, MA). Blood samples were processed by centrifugation at 1,500 x *g* for 15 min, aliquoted into 2 ml cryogenic tubes (BD Falcon™, Franklin Lakes, NJ), and stored at -20°C until tested.

Mammary secretions

Colostrum and milk samples for antibody testing were collected from sows daily between DPIs -3 to 14. Sows were administered 20 USP units of oxytocin (VetOne®) to facilitate collection of mammary secretions. Samples were processed by centrifugation at 13,000 x *g* for 15 min at 4°C to remove fat and debris. The defatted samples were then aliquoted into 2 ml cryogenic tubes (BD Falcon™) and stored at -20°C until tested.

Fecal samples

Fecal samples for porcine coronavirus RT-PCR testing included fecal swab (BD BBL™ CultureSwab™ Collection/Transport system, Thermo-Fisher Scientific) samples collected from individual sows immediately prior to receipt of the animals and individual piglet fecal samples collected between DPIs 0 and 14. Approximately 1 gram of feces was collected from each piglet using a disposable fecal loop (VetOne®), mixed with 1 ml PBS (1X pH 7.4,

Sigma-Aldrich) immediately after collection, placed in a 2 ml cryogenic tube (BD Falcon™), and stored at -80°C.

Coronavirus reverse-transcriptase polymerase chain reactions (rRT-PCR)

RNA extraction

In brief, viral RNA was extracted from 100 µl of fecal swab samples and eluted into 90 µl of elution buffer using the Ambion® MagMAX™ viral RNA isolation kit (Life Technologies) and a KingFisher® 96 magnetic particle processor (Thermo-Fisher Scientific) following the procedures provided by the manufacturers.

Coronavirus primers and probes

Sow fecal swab samples and piglet fecal samples were tested for PEDV using a PEDV nucleocapsid (N) gene-based rRT-PCR described in Madson et al. (2014) and performed routinely at the Iowa State University-Veterinary Diagnostic Laboratory (ISU-VDL SOP 9.5263). Primers and probes targeting conserved regions of the PEDV N gene were designed to match a U.S. PEDV nucleotide sequences published in GenBank® (accession no. KF272920) (Madson et al., 2014).

Sow fecal swab samples were tested for TGEV using a spike (S) gene-based rRT-PCR described in Kim et al. (2007) and performed routinely at the ISU-VDL (ISU-VDL SOP 9.5575). Primers and probes targeting conserved regions of the TGEV S gene were designed to match 9 TGEV strains, including Purdue 46-MAD (GenBank® NC00236), TO14 (GenBank® AF302264), TS (GenBank® DQ201447), SC-Y (GenBank® DQ443743), Miller M6 (GenBank® DQ811785), TH-98 (GenBank® AY676604), HN2002 (GenBank® AY587884), 96-1993 (GenBank® AF104420), and FS772/70 (GenBank® Y00542) (Kim et al., 2007).

Sow fecal swab samples were tested for PDCoV using a membrane (M) gene-based rRT-PCR described in Chen et al. (2015) and performed routinely at the ISU-VDL (ISU-VDL SOP 9.5478). The protocol included positive control standards of known infectivity titers ($TCID_{50}$). In brief, the forward primer, reverse primer, and probe were designed to match the

M gene of global and U.S. PDCoV isolates. The probe was labeled with FAM/ZEN/3'Iowa Black Detector (Integrated DNA Technologies, Coralville, IA).

Real-time RT-PCR

The eluted RNA, primers, and probe were mixed with commercial reagents (Path-ID® Multiplex One-Step RT-PCR kit, Life Technologies) and the RT-PCR reactions were conducted on an ABI 7500 Fast instrument (Life Technologies) as follows: 48°C for 10 min, 95°C for 10 min, 95°C for 15 s (45 cycles) and 60°C for 45 s. The real-time RT-PCR (rRT-PCR) results were analyzed using an automatic baseline setting with a threshold at 0.1. Quantification cycle (Cq) values ≤ 35 were considered positive for the corresponding coronavirus. Data were reported as "adjusted Cq":

Equation 1.

$$Adjusted\ Cq = (35 - sample\ Cq)$$

Coronavirus antibody assays

PEDV indirect immunofluorescence assay (IFA)

IFA plates were prepared by inoculating confluent monolayers of Vero cells (ATCC® CCL-81™) in 96-well plates (CoStar™, Corning®) with 100 µl/well of PEDV (USA/IN19338/2013) at 1 x 10^3 plaque-forming units/ml. The plates were then incubated for 18 to 24 h, after which the inoculum was removed and the cell monolayers fixed with cold acetone:alcohol (70:30) solution (Sigma-Aldrich). Plates were then air-dried, sealed, and stored at -20°C. To perform the test, serum samples were serially two-fold diluted (1:40 to 1:320) in PBS (1X pH 7.4) and then 100 µl of each dilution was transferred to IFA plates and incubated at 37°C for 1 h. After incubation, the diluted serum samples were removed from test plates, the plates rinsed 3 times with PBS (1X pH 7.4) and 50 µl of 1:50 diluted with fluorescein isothiocyanate (FITC) labeled goat anti-swine polyclonal antibody (Abcam®, Cambridge, MA) was added to each well. After a 30 min incubation at 37°C, the plates were rinsed again with PBS (1X pH 7.4) and the cells were observed under an inverted fluorescent microscope for PEDV-specific cytoplasmic staining.

PEDV whole virus antibody ELISA

PEDV (USA/NC35140/2013) was used in the PEDV whole-virus based antibody ELISA. In brief, virus was propagated on Vero cells, the flasks subjected to one freeze-thaw, and the harvested material centrifuged at 4,000 x *g* for 15 min to remove cell debris. The virus was then pelleted by ultracentrifugation at 140,992 x *g* for 3 h, after which the virus pellet was washed twice with sterile PBS (1X pH 7.4). The purified virus was resuspended in PBS (1X pH 7.4) at a dilution of 1:100 of the original supernatant volume and stored at -80°C. Following titration and optimal dilution, polystyrene 96-well microtitration plates (Nalge Nunc Corp.) were manually coated (100 µl per well) with the viral antigen solution and incubated at 4°C overnight in a closed container containing a towel saturated with water. After incubation, plates were washed 5 times, blocked with 300 µl per well of a blocking solution containing 1% bovine serum albumin (Jackson ImmunoResearch Inc., West Grove, PA), and incubated at 25°C for 2 h. Plates were then dried at 37°C for 4 h and stored at 4°C in a sealed bag with desiccant packs. Plate lots with a coefficient of variation ≥ 10% were rejected.

ELISA conditions for the detection of anti-PEDV IgA and IgG antibodies in serum and colostrum/milk (defatted) specimens, including coating and blocking conditions, reagent concentrations, incubation times, and buffers, were identical. High positive, low positive, and negative plate controls, i.e., antibody-positive and -negative experimental serum or milk samples, were run in duplicate on each ELISA plate. All samples were diluted 1:50, after which plates were loaded with 100 µl of the diluted sample per well. Plates were incubated at 25°C for 1 h and then washed 5 times with PBST wash solution (PBS 1X , 0.1% Tween-20, pH 7.4).

To perform the assay, 100 µl of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories Inc., Montgomery, TX) diluted 1:20,000 for serum and colostrum/milk samples or goat anti-pig IgA (Bethyl Laboratories Inc.) diluted 1:3,000 for serum and 1:45,000 for colostrum/milk samples was added to each well and the plates incubated at 25°C for 1 h. After a washing step, the reaction was visualized by adding 100 µl of tetramethylbenzidine-hydrogen peroxide (TMB, Dako North America, Inc., Carpinteria, CA)

substrate solution to each well. After 5 min incubation at room temperature, the reaction was stopped by the addition of 50 µl of stop solution (1 M sulfuric acid) to each well. Reactions were measured as optical density (OD) at 450 nm using an ELISA plate reader (Biotek® Instruments Inc., Winooski, VT) operated with commercial software (GEN5™, Biotek® Instruments Inc.). The antibody response in serum and colostrum/milk samples was represented as sample-to-positive (S/P) ratios:

Equation 2.

$$S/P\ ratio = \frac{(sample\ OD - blank\ well\ control\ mean\ OD)}{(positive\ control\ mean\ OD - blank\ well\ control\ mean\ OD)}$$

For serum, S/P ratios ≥ 0.80 were considered positive for PEDV IgG antibody (Bjustrom-Kraft et al., 2016).

PEDV fluorescent focus neutralization (FFN) assay

Colostrum, milk, and serum samples were tested for neutralizing antibody. Prior to FFN testing, defatted milk and colostrum samples were treated with Rennet (Rennet from *Mucor miehei*, Sigma-Aldrich). In brief, 5 µl Rennet was added to 1 ml of defatted milk or colostrum and briefly vortexed. The mixture was then incubated at 37°C for 30 min, vortexed, and then centrifuged at 2,000 *x g* for 15 min. The supernatant was then harvested and tested for neutralizing antibody.

To perform the FFN, test samples, antibody-positive control serum, and antibody-negative control serum were heat inactivated at 56°C for 30 min and then 2-fold serially diluted (1:4 to 1:512) in 96-well dilution plates (Axygen®, Corning®) using post-inoculation medium to give a final volume of 100 µl. Then, 75 µl of each dilution was transferred to new dilution plate (Axygen®, Corning®), mixed with 75 µl of PEDV (1 x $10^{3.6}$ $TCID_{50}$/ml) to give final serum dilutions of 1:8 to 1:1024, and incubated at 37°C with 5% CO_2 for 1 h. Vero cell confluent monolayers in 96-well plates (CoStar™, Corning®) were washed twice with post-inoculation medium, inoculated with 100 µl of the sample-virus mixture, incubated at 37°C

with 5% CO_2 for 1 h, and washed twice. 100 µl of post-inoculation medium was then added to each well and the plates incubated at 37°C with 5% CO_2 for 48 h. Finally, cells were fixed with 80% cold acetone:alcohol (80:20), stained with FITC-conjugated monoclonal antibody (SD6-29, Medgene Labs, Brooking, SD) for 1 h, and observed under an inverted fluorescent microscope for PEDV-specific cytoplasmic staining. Positive neutralizing endpoints were determined as the highest dilution resulting in a ≥ 90% visual reduction in fluorescing foci relative to the antibody-negative serum control. Plates in which the positive control deviated more than 2-fold from its expected antibody titer were considered invalid.

Analysis

Statistical analyses were performed using SAS® 9.4 (SAS® Institute Inc., Cary NC, USA). Body weight, percent change in body weight, body temperature, and fecal shedding (PEDV rRT-PCR Cq) were analyzed using linear mixed models. Treatment, DPI, and their interaction were analyzed as fixed effects and piglet and sow were analyzed as random effects. The time to death was analyzed using proportional hazard regression analyses, with a robust sandwich covariance matrix estimate to account for the dependence within the same sow. When no statistical significance was shown in the 6 group model, a 2 group (antibody-positive vs. antibody-negative) model was run.

Percent change in body weight for any DPI was calculated relative to DPI -4:

Equation 3.

$$Percent\ change = \frac{(weight - weight\ DPI\text{-}4)}{(weight\ DPI\text{-}4)} \times 100$$

Normal body temperature was defined as values within the 95% confidence interval calculated for the body temperature data collected on DPIs -4 and -1. The qualitative effect of PEDV infection body temperature was assessed by analyzing the proportion of piglets within the "normal range" of body temperature over time post inoculation using one-way ANOVA. The number of animals with normal body temperature (yes/no) and PEDV fecal shedding (yes/no) of each group were compared using one-way ANOVA.

Differences among sows in the number of surviving piglets were compared using the Kruskal-Wallis test. Differences among treatments in the time to death were analyzed using proportional hazard regression analysis with a robust sandwich covariance matrix estimate to account for dependence within the same sow.

Results

Sows

Sow fecal samples collected prior to inoculation were qRT-PCR-negative for PEDV, TGEV, and PDCoV. Likewise, serum samples collected from sows at DPI -7 were antibody-negative by IFA (<1:8) and PEDV WV ELISA (S/P <0.80). On this basis, all sows were considered PEDV-naïve at the time the experiment commenced.

The 7 sows farrowed a total of 74 liveborn pigs, 3 stillborn pigs, and 4 mummified fetuses (Table 1). Each piglet litter was kept intact and remained with its dam throughout the study. One sow and her litter were eliminated from the study on DPI 6 because the sow was agalactic. The remaining 6 sows were clinically normal throughout the study. No significant difference in number of survival piglets was detected among the 6 litters (Kruskal-Wallis test).

Sow serum samples collected on DPI -7 had PEDV WV IgG ELISA S/P ratios between 0.2 and 0.6. By DPI 14, S/P ratios ranged from 1.2 to 2.7. Sow milk samples collected on DPI -5 had PEDV WV IgA ELISA S/P ratios between 0.0 and 0.2. By DPI 14, milk S/P ratios ranged between 0.4 and 2.0 (Fig. 1). Rising anti-PEDV antibody levels in serum and milk indicate that the sows were infected with PEDV over the course of the experiment, presumably by exposure to PEDV-contaminated piglet feces.

Piglets

Prior to PEDV inoculation, all piglets were clinically normal in appearance and behavior. Piglets were confirmed free of PEDV infection prior to inoculation on the basis of negative qRT-PCR results on individual piglet fecal samples collected on DPI 0.

All piglets had normal feces on the day of inoculation, but 29% (n = 16), 69% (n = 43), and 89% (n = 50) of piglets had semi-solid or watery feces on DPIs 1, 2, and 3, respectively. A gradual resolution in the diarrhea was observed thereafter. Fecal loop samples from ≥ 1 pigs in each treatment group were PEDV rRT-PCR positive on DPI 1, with all samples positive on DPI 2. The highest concentration of virus in feces was observed between DPI 2 to 4 (Fig. 2a). All fecal samples from piglets in the antibody-negative control group were positive through DPI 12. Fecal samples from the single remaining piglet in this group were negative on DPIs 13 and 14. PEDV rRT-PCR negative fecal samples were observed in the antibody-positive groups beginning on DPI 6, but positive fecal samples were recovered from 40% (n = 6) of antibody-positive pigs on DPI 14. Statistical analysis found no effect of treatment on the quantity of virus shed with treatment defined as 6 groups or defined as 2 groups (antibody-positive and antibody-negative control).

At the time of PEDV inoculation, mean piglet weight by treatment ranged from 1.9 to 2.3 kg. Piglets who survived to the end of the study gained 12.5 to150.0% of their body weight. No significant difference in the effect of PEDV on body weight was detected either with treatment defined as 6 groups (5 different PEDV antibody concentrations and an antibody-negative control) or with treatment defined as 2 groups (antibody-positive and antibody-negative control).

The quantitative effect of PEDV infection on body temperature over time is shown in Fig. 2b. No significant difference in body temperature was detected with treatment defined as 6 groups, but a comparison based on 2 groups (antibody-positive vs antibody-negative) showed a significant difference in body temperature between the two groups on DPIs 4, 5, 6, and 8 (linear mixed model, $p < 0.05$).

The qualitative effect of PEDV infection body temperature was assessed by analyzing the proportion of piglets with "normal" body temperature (yes/no). Body temperature data collected on DPIs -4 to -1 showed a mean piglet body temperature of 39.3^0C with a 95% confidence interval of 38.6^0C to 40.1^0C ("normal range"). All piglets were within the normal

range on DPI 0. Piglets with body temperature below the lower bound of the normal range were observed from DPI 1 through 12. All survivor piglets had body temperatures within the normal range on DPIs 13 and 14. A statistical analysis of the body temperature data found no effect of treatment on the proportion of piglets with normal body temperature either with treatment defined as 6 groups or defined as 2 groups.

In total, 71% piglets (n = 44) died between 2 and 13 DPI (Fig. 2c). Of these, 22.7% (n = 10) were humanely euthanized because they met previously established clinical criteria (unable to suckle, reluctant to stand, or ≥ 10% dehydration). Compared to the antibody-negative control group, a significant difference in time to death (treatment 5, $p < 0.05$), or a trend toward a difference (treatment 6, $p = 0.11$) was detected by hazard regression analysis.

As shown in Table 2, piglet serum samples collected on DPI -4 had FFN antibody titers <1:8, ELISA IgA S/P ratios ranging from 0.5 to 0.7, and ELISA IgG S/P ratios between 0.6 and 0.7 (IgG). Depending on the treatment, serum samples collected on DPI 0, i.e., 24 h post treatment, had FFN antibody titers ranging from <1:8 to 1:32 and ELISA S/P ratios of 0.2 to 3.3 (IgA) and 0.5 to 1.4 (IgG). Given the number of sampling points (DPIs -4, 0, 14) and the small sample size (number of surviving piglets) at the termination of the study, the PEDV antibody response on DPIs -4, 0, and 14 was analyzed using the Wilcoxon rank test. Using this approach, no significant differences in FFN, IgA, and IgG serum antibody responses were detected in treated versus control groups on DPIs -4 and 14, but most treatment groups had significantly higher antibody responses relative to antibody-negative piglets (treatment 1) on 0 DPI (Table 2).

Discussion

The objective of this experiment was to expand our understanding of immunity against PEDV by quantifying the effect of circulating passive antibody on the course of PEDV infection in neonatal piglets using a "passive antibody model". Physiologically, this approach relied on the fact that antibodies injected into the peritoneum are quickly taken up by the lymphatic system and enter the circulatory system via the vena cava (Harlow and Lane, 1988). Essentially, intraperitoneal injection gives the same bioavailability as

intravenous injection. Thus, it was possible to produce different levels of circulating PEDV antibody by intraperitoneal inoculation with specific levels of concentrated PEDV antibody solution (Table 2).

The passive antibody model has previously been used to study maternally-derived humoral immunity in mice (Briles et al., 2000; Traggiai et al., 2004; Subbarao et al., 2004), rats (Walsh et al., 1984), and hamsters (Guillaume et al., 2004). In swine, this method was previously used to study the effect of passive antibody on rotavirus infection (Hodgins et al., 1999; Nguyen et al., 2006a,b; Parreno et al., 1999) and porcine reproductive and respiratory syndrome virus (PRRSV; Yoon et al., 1996; Lopez et al., 2007). Hodgins et al. (1999) found that passive antibody provided protection against clinical rotaviral infection, but also suppressed the piglets' active immune responses. Nguyen et al. (2006) reported that passive antibody protected neonates against rotavirus and determined that high titers of maternal antibody suppressed effector and memory B-cell responses. Other workers found that intraperitoneal injection of PRRSV antibody sufficient to achieve serum neutralizing antibody titers of $\geq$ 1:16 inhibited PRRSV replication in 2 -to 5-week-old pigs (Yoon et al., 1996; Lopez et al., 2007).

In the current experiment, the effect of passive antibody on outcomes associated with PEDV infection, e.g., body weight, body temperature, survival, PEDV shedding in piglet feces, and serological responses to infection, was evaluated over a range of antibody treatment levels. The size of the experiment was limited by the number of piglets within treatments and the physical and logistical requirements of daily observations, sampling, and handling. The limitations of the experiment were offset by the experimental design. Specifically, the randomized block design allowed for random allocated of all treatments to all litters, thereby controlling for differences among sows.

In neonatal piglets inoculated with PEDV under experimental conditions, diarrhea typically occurs within one DPI (Coussement et al., 1982; Kim and Chae, 2002), PEDV is detected in feces within 2 DPI (Jung et al., 2014; Madson et al., 2014; Thomas et al., 2015), and mortality commences within 3 DPI (Shibata et al., 2001). A similar pattern was observed

under the conditions of this experiment. Diarrhea was first observed at one DPI, all fecal samples were PEDV rRT-PCR positive on DPI 2, and mortality commenced on DPI 2. Statistical analyses found that circulating PEDV antibody did not protect piglets from the negative effects of PEDV on growth, reduce or eliminate shedding of PEDV in feces, or affect the humoral immune response against PEDV infection. However, circulating antibody partially ameliorated the effect of PEDV infection on body temperature and improved piglet survivability. Specifically, antibody-positive groups returned to normal body temperature faster (Fig. 2b) and demonstrated higher survivability than PEDV antibody-negative control piglets (Fig. 2c). These results are compatible with previous reports for swine coronaviruses. Shibata et al. (2001) reported that passive PEDV-specific antibody was effective in preventing PEDV infection and reduced mortality in 2 day-old piglets. Stepanek et al. (1982) demonstrated that the presence of sufficient levels of TGEV-specific passive antibody delayed mortality due to TGEV infection in 4 day-old pigs. The mechanisms responsible for producing these results have not been established, but we hypothesize that one or more of the following four mechanisms may be involved:

1. Although viremia was not confirmed in this experiment, a PEDV viremia lasting at least 7 DPI has been reported in young pigs inoculated under experimental conditions (Opriessnig et al., 2014). Since piglets administered concentrated antibody had FFN antibody titers of up to 1:32 at 0 DPI, it may be hypothesized that circulating neutralizing antibodies delivered via intraperitoneal administration may have reduced the level and/or the duration of PEDV viremia and modified the clinical course of the infection. There are no reports on PEDV with which these results can be compared, but the results would be consistent with a previous report by Lopez et al. (2007) showing that intraperitoneal administration of PRRSV-neutralizing antibody reduced or eliminated PRRSV viremia in young pigs (15 day-old) and delayed transmission to commingled sentinel pigs.

2. During PEDV viremia, binding of circulating PEDV antibodies (neutralizing and non-neutralizing) to viral antigenic determinants may have resulted in neutralization, agglutination, and/or complement fixation. This process could have facilitated the humoral and/or cellular immune responses by presenting antigen to the appropriate cells

(dendritic cells, macrophages, and B cells) (Unanue, 1984; Steinman, 1991; Guermonprez et al., 2002).

3. Antibody-dependent cell-mediated cytotoxicity (ADCC) effected by interactions between antibody and other components of the immune system, e.g. complement, phagocytic cells, and natural killer cells, could have expedited cell-mediated immune responses (CMI) against PEDV (Casadevall and Pirofski, 2003). ADCC kills antibody-coated infected cells by inducing the expression of cell death-inducing molecules (Henkart, 1985). Presumably, this mechanism is effective for any PEDV-infected cells; not only enterocytes. Madson et al. (2015) has reported that, in addition to enterocytes, cells in the mesenteric lymph nodes and spleen may stain positive for PEDV by immunohistochemistry.

4. Passively-transferred, circulating PEDV IgG could have passed directly from capillaries into the small intestine by paracellular leakage and neonatal Fc receptors (Hodgins et al. 1999; Parreno et al., 1999; Neutra and Kozlowski, 2006). If so, the transported IgG may have neutralized PEDV in the intestinal lumen and/or assisted the humoral and CMI responses by facilitating uptake of PEDV antigen through receptors on apical surfaces of microfold cells (Yoshida et al., 2006; Neutra and Kozlowski, 2006). Again, there is no PEDV research against which to test this hypothesis, but IgG is known to play an important role against parvovirus infection in crypt cells (Hoare et al., 1997). Evidence against this hypothesis is the fact that IgA is believed to play a primary role in protecting against enteric viruses that infect villous enterocytes, e.g. TGEV and rotavirus (Chattha et al., 2015), and PEDV primarily infects villous enterocytes (Madson et al., 2015).

Combining the results of this experiment with previous work reported in the literature leads to the conclusion that both colostral antibody and maternal secretory IgA in milk contribute to the protection of the neonatal pig against PEDV infections. Beyond this observation lies a list of questions, e.g., What mechanism(s) effect protection? Which antibody isotype(s) and at what concentration are protective? How do the antibody levels and antibody isotypes examined in this experiment relate to those achieved in production settings by feedback,

vaccination, or a combination of the two? Regardless, it is clear that the optimal protection to piglets will be provided by dams able to deliver both high antibody titers in colostrum and high titers of secretory IgA in milk.

Funding

This study was funded in part by Checkoff Dollars administered through the National Pork Board, Des Moines, Iowa (USA).

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Tables

Table 1. Allocation of piglets to treatments by litter[a]

Litter	Age (days) at time of treatment	No. of piglets	Groups (no. piglets within treatment)[b]					
			1[b]	2	3	4	5	6
1	5	13	2	3	2	2	2	2
2	4	11	2	1	2	2	2	2
3	4	9	1	2	1	2	2	1
4	4	10	2	1	2	1	1	3
5	4	10	2	2	1	2	2	1
6	4	9	2	2	2	1	1	1
7[c]	2	12	1	1	3	3	2	2
Totals		74	12	12	13	13	12	12

[a]Piglets were assigned to treatment using randomized block design whereby all treatments were assigned to each litter.

[b]Treatment 1 piglets served as negative controls. Piglets in treatments 2 to 6 were administered increasing levels of antibody (see Table 2).

[c]Litter 7 was excluded from the study because the sow was agalactic.

Table 2. Serum antibody levels among treatment groups by day post inoculation

Assay	Group	Day post inoculation		
		-4	0[a]	14
FFN arithmetic mean (SE)	1	<1:8 (4.6)	<1:8 (4.6)	1:64 (6.1)
	2	<1:8 (4.6)	1:5.3 (4.6)	1:19.7 (4.9)
	3	<1:8 (4.6)	1:6.1 (4.6)[b]	1:19.7 (4.9)
	4	<1:8 (4.6)	1:8.0 (4.6)[b]	1:32.0 (5.3)
	5	<1:8 (4.6)	1:17.1 (4.6)[b]	1:11.3 (4.9)
	6	<1:8 (4.6)	1:32.0 (4.6)[b]	1:16.0 (5.3)
PEDV IgA ELISA least square mean S/P (SE)	1	0.5 (0.2)	0.2 (0.2)	2.2 (0.4)
	2	0.7 (0.2)	0.7 (0.2)	2.0 (0.2)
	3	0.6 (0.2)	1.1 (0.2)	1.9 (0.3)
	4	0.6 (0.2)	1.8 (0.2)[b]	1.3 (0.3)
	5	0.6 (0.2)	2.8 (0.2)[b]	1.2 (0.2)
	6	0.7 (0.2)	3.3 (0.2)[b]	1.4 (0.3)
PEDV IgG ELISA least square mean S/P (SE)	1	0.6 (0.1)	0.5 (0.1)	1.7 (0.2)
	2	0.7 (0.1)	0.7 (0.1)[b]	1.0 (0.1)
	3	0.7 (0.1)	0.7 (0.1)[b]	1.5 (0.2)
	4	0.7 (0.1)	0.8 (0.1)[b]	1.3 (0.2)
	5	0.7 (0.1)	1.1 (0.1)[b]	1.0 (0.1)
	6	0.7 (0.1)	1.4 (0.1)[b]	0.9 (0.2)

[a]24 hours following intraperitoneal administration of concentrated PEDV antibody

[b]Significantly different from Group One (Wilcoxon rank test, $p < 0.02$)

Figures

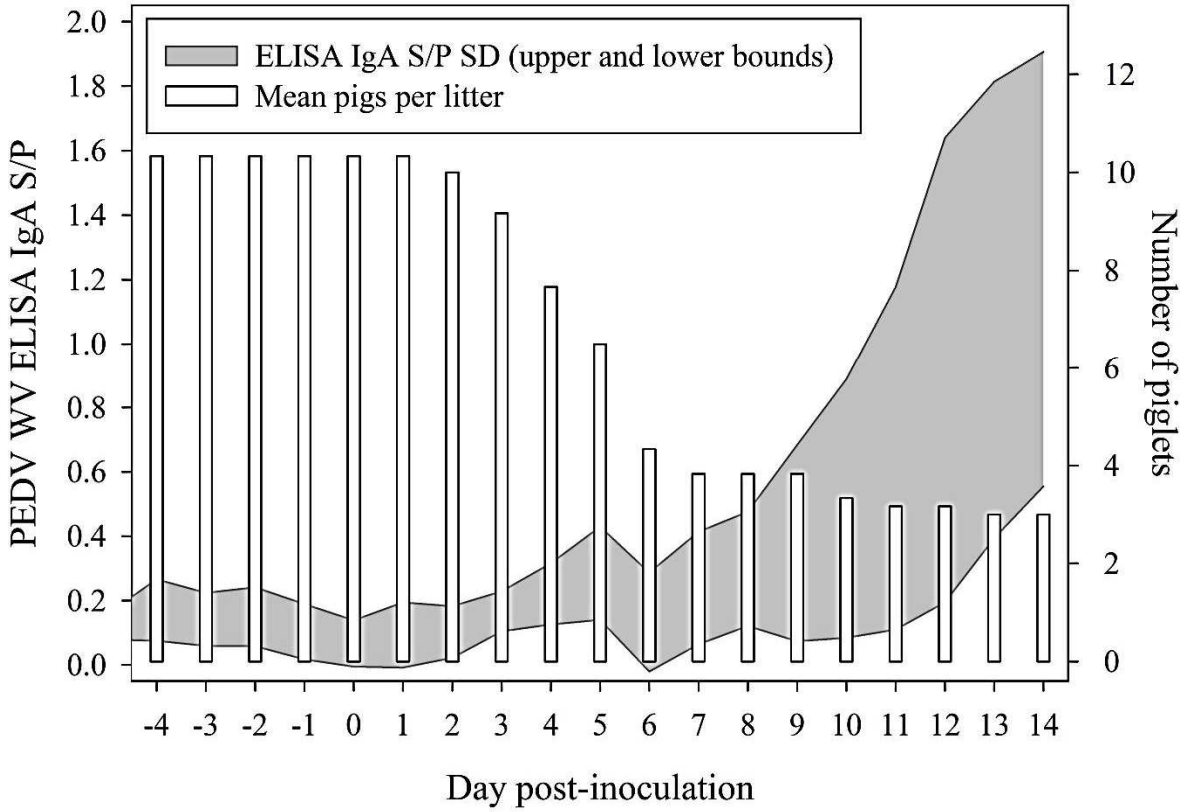

Figure 1. Anti-PEDV IgA in milk (standard deviation upper and lower bounds) based on daily samplings and mean number of piglets per litter (n = 6) over time post inoculation. No significant difference in survival was detected among the 6 litters (Kruskal-Wallis test).

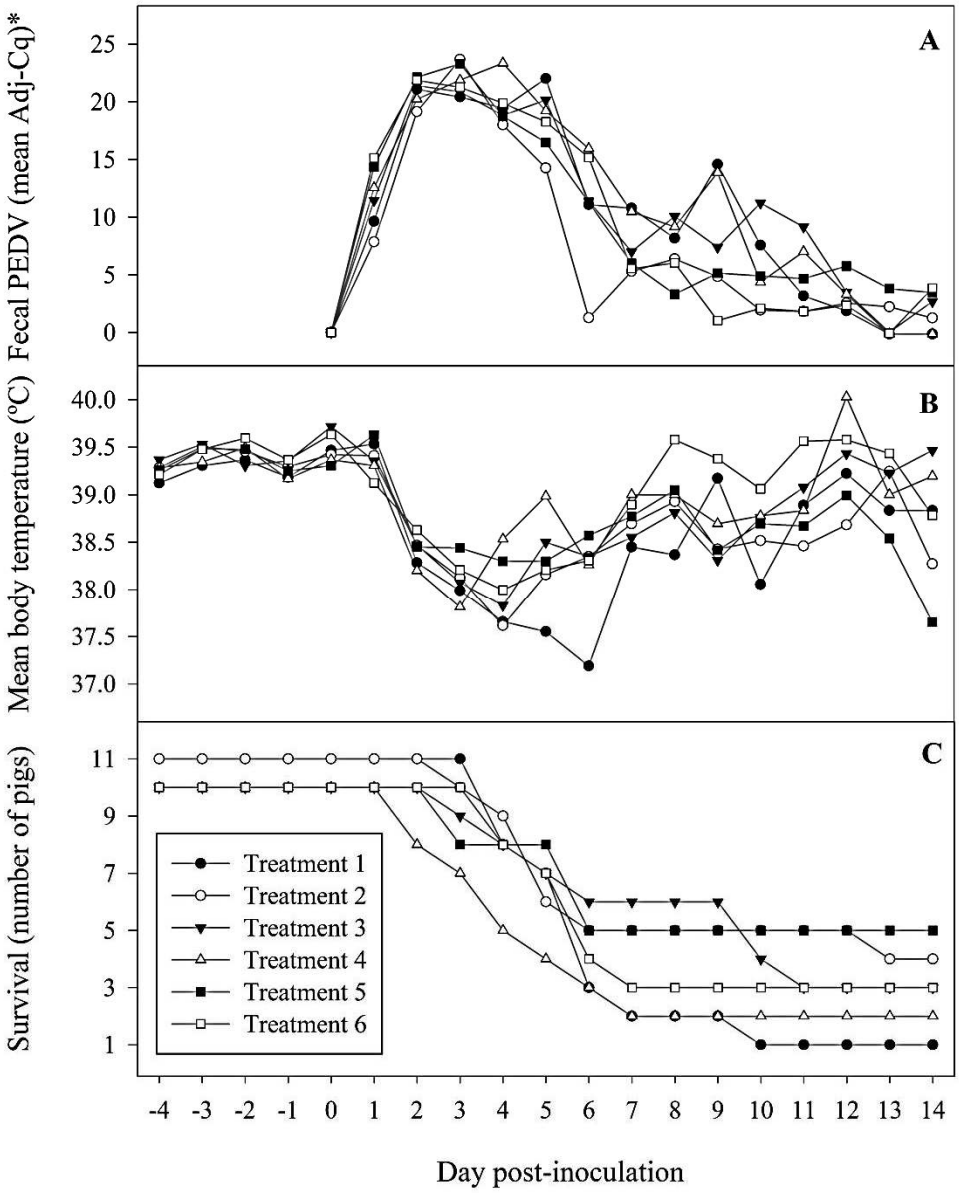

Figure 2. Treatment responses following inoculation of piglets with porcine epidemic diarrhea virus (USA/IN/2013/19338E). Treatment 1 piglets served as antibody-negative controls; piglets in treatments 2 to 6 had increasing levels of circulating anti-PEDV antibody. [a]Adjusted PEDV rRT-PCR quantification cycle (Cq) = (35 – sample Cq)

CHAPTER 4. QUANTIFYING THE EFFECT OF LACTOGENIC ANTIBODY ON PORCINE EPIDEMIC DIARRHEA VIRUS INFECTION IN NEONATAL PIGLETS

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Modified from a manuscript published in Veterinary Microbiology 197: 83-92

Abstract

The contribution of lactogenic antibody to the protection of piglets against porcine epidemic diarrhea virus (PEDV) was evaluated. Pregnant multiparous sows and their litters were allocated to one of 3 treatment groups: Group 1 - 6 serum antibody-negative sows and a subset (n = 11) of their piglets. Group 2 - 8 serum antibody-positive sows and their 91 piglets. Piglets were orally inoculated with PEDV at 4 (Group 1) or 2 (Group 2) days of age. Group 3 - 2 PEDV serum antibody-negative sows and 22 piglets, provided a baseline for piglet survivability and growth rate. Piglets were monitored daily for clinical signs, body weight, and body temperature through day post-inoculation (DPI) 12 (Groups 2 and 3) or 14 (Group 1). Serum and mammary secretions were tested for PEDV IgG, IgA, and virus-neutralizing antibody. Feces were tested by PEDV real-time, reverse transcriptase PCR (rRT-PCR). Piglets on sows without (Group 1) or with (Group 2) anti-PEDV antibody showed significantly different responses to PEDV infection in virus shedding ($p < 0.05$), thermoregulation ($p < 0.05$), growth rate ($p < 0.05$), and survivability ($p < 0.0001$). Specifically, Group 1 piglets shed more virus on DPIs 1 to 5, were hypothermic at all sampling points except DPIs 9, 11, and 12, gained weight more slowly, and exhibited lower survivability than Group 2 piglets. Within Group 2 litters, significant differences were found

in virus shedding ($p < 0.05$), and body temperature ($p < 0.05$), but not in piglet survival rate. The number of sows and litters in Group 2 was insufficient to derive the relationship between specific levels of lactogenic antibody (FFN, IgA, and IgG) and the amelioration of clinical effects. However, when combined with previous PEDV literature, it can be concluded that the optimal protection to piglets will be provided by dams able to deliver sufficient lactogenic immunity, both humoral and cellular, to their offspring.

Introduction

Porcine epidemic diarrhea virus (PEDV), a member of family *Coronaviridae,* is a cause of diarrhea and vomiting in pigs of any age (Pensaert and de Bouck, 1978; Pospischil et al., 2002; Wood, 1977). In neonatal piglets, PEDV can cause severe diarrhea and vomiting, thereby leading to dehydration, metabolic acidosis, and death (Pospischil et al., 2002; Pensaert, 1999). Clinical outbreaks of porcine epidemic diarrhea were first reported in 1971 in England, with its viral etiology determined in 1978 (Pensaert and de Bouck, 1978; Wood, 1977). Previously free of the virus, outbreaks of PEDV in North America were first reported in April 2013 (Alvarez et al., 2016; Stevenson et al., 2013). Spreading rapidly across the continent, PEDV caused a 6 to 7% decline in U.S. pork production in 2014 (Alvarez et al., 2015).

The impact of PEDV infection is age-dependent. In piglets, the greater severity of PEDV is the result of slower villus-epithelial repopulation and the immaturity of the neonatal immune system (Annamalai et al., 2015; Jung et al., 2015; Jung and Saif, 2015; Madson et al., 2014; Moon, 1971; Shibata et al., 2000). Thus, the introduction of PEDV into a naïve swine farm can result in ≥ 90% mortality in piglets less than 2 weeks of age and up to 40% mortality in 2- to 4-week-old pigs (Stevenson et al., 2013). PEDV has also been reported to impact reproductive performance in pregnant gilts and sows. Olanratmanee et al. (2010) reported a 12.6% decrease in the farrowing rate, 5.7% increase in the rate returns, a 1.3% increase in the abortion rate, and a 2.0% increase in mummified fetuses per litter following an outbreak of PEDV in a large production system.

In the field, it has been observed that prior exposure of the sow herd to PEDV ameliorates the impact of PEDV infection in neonates, presumably via maternal immune mechanisms (Goede et al., 2016). Based on these observations, it has been postulated that PEDV-specific IgG and IgA in colostrum and milk are critical for the protection of neonatal piglets against PEDV (Chattha et al., 2015; Song et al., 2015). Using TGEV as a model, the assumption is that lactogenic immunity against PEDV is induced by enteric infection following oral exposure (Song et al., 2007). Thereafter, IgA plasmablasts stimulated in the sow's gut migrate to the intestinal lamina propria and then to the mammary glands where they produce PEDV-specific IgA (Bohl et al., 1972a; Saif et al., 1972). Lactogenic IgA is effective within the intestinal tract of the neonatal pig because it has high affinity and is resistant to proteolysis (Song et al., 2015). However, as discussed by Langel et al. (2016), there are no published reports on the role of lactogenic antibody in the protection of piglets against PEDV. Therefore, the objective of this experiment was to quantify the effect of lactogenic immunity in PEDV-inoculated piglets by comparing viral shedding, piglet growth, thermoregulation, and survival in litters with PEDV-immune vs naïve dams.

Materials and methods

Experimental design

The study was conducted under the approval of the Iowa State University Office for Responsible Research. Pregnant multi-parous sows (n = 16), 8 PEDV serum antibody-negative and 8 PEDV serum antibody-positive, were farrowed in biosafety level 2 (BSL-2) research facilities. Sows and their litters were allocated to one of 3 treatment groups: Group 1 (positive controls) consisted of 6 PEDV serum antibody-negative sows and 11 of their 74 piglets. The remaining piglets from these litters were used in an experiment described elsewhere (Poonsuk et al., 2016). Group 1 piglets provided a baseline response for PEDV infection in the absence of maternal immunity. Group 2 consisted of 8 PEDV serum antibody-positive sows and 91 piglets. Group 2 provided a measure of the effect of maternal immunity. Group 3 consisted of 2 PEDV serum antibody-negative sows and 22 piglets. This group provided a baseline for piglet survivability and growth rate.

Piglets were orally inoculated with PEDV at 4 (Group 1) or 2 (Group 2) days of age; sows were not inoculated, but had contact with PEDV-contaminated piglet feces. All piglets were closely observed for clinical signs through day post-inoculation (DPI) 12 (Groups 2 and 3) or 14 (Group 1) or until humane euthanasia was necessary. Body weight and body temperature measurements were taken daily. Sow mammary secretions and piglet fecal samples were also collected daily. Serum samples were collected from sows prior to farrowing and at the termination of the experiment, i.e., DPI 12 (Groups 2 and 3) or 14 (Group 1). Serum samples were collected from piglets at DPI 0 and either at the time of euthanasia or the termination of the experiment. Serum, colostrum, and milk were tested for PEDV IgG, IgA, and virus-neutralizing antibody. Feces were tested by PEDV real-time, reverse transcriptase PCR (rRT-PCR). Data were analyzed for the effect of maternal PEDV antibody levels in colostrum and milk on piglet PEDV serum antibody levels, PEDV fecal shedding, body temperature, weight gain, and mortality.

Animals and animal care

Sixteen clinically healthy, multiparous sows were acquired from 2 commercial sow farms between 84 to 110 days of gestation. To verify their PEDV status, serum samples collected on DPI -7 were tested for PEDV antibody by PEDV fluorescent focus neutralization (FFN) assay and a PEDV whole-virus indirect ELISA. To verify the absence of acute coronavirus infection, fecal swabs were collected immediately prior to receipt of animals and tested for PEDV, transmissible gastroenteritis virus (TGEV), and porcine delta coronavirus (PDCoV) using agent-specific qRT-PCRs. Animals were housed throughout the experiment in the Iowa State University Livestock Infectious Disease Isolation Facility (LIDIF), a BSL-2 research facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The facility is equipped with a single-pass non-recirculating ventilation system that provided directional flow from low contamination areas to high contamination areas and zones of negative pressure to prevent airborne contamination from area-to-area or room-to-room. Each room was ventilated separately and humidity and temperature was strictly controlled. Animals were closely observed (multiple times daily) from the time they entered LIDIF to the end of the observation period by researchers, animal caretakers, and veterinary staff.

Sows were housed two per room in Danish-style free stall farrowing crates (Thorp Equipment Inc., Thorp, WI) and supplemental heat was provided for piglets. To induce parturition, all sows were administered 10 mg of dinoprost tromethamine (Lutalyse®, Zoetis Inc., Florham Park, NJ) 24 hours prior to the expected farrowing date, i.e., on day 113 of gestation. The piglets included in the study (n = 124) were ear-tagged and administered 1 ml iron hydrogenated dextran (VetOne®, Boise, ID) and 5 mg (0.1 ml) ceftiofur sodium (Excenel®, Zoetis). Piglets remained on the sow continuously throughout the observation period.

Porcine epidemic diarrhea virus (PEDV) inoculum

The PEDV isolate used in the study (USA/IN/2013/19338E) was isolated in 2013 at the Iowa State University Veterinary Diagnostic Laboratory from piglet small intestine submitted from an Indiana swine farm (Chen et al., 2014). The inoculum used in this study was the 7th passage of the virus on cell culture. For this study, the virus was serially propagated on Vero cells (African green monkey kidney) in flasks using methods described elsewhere (Hofmann and Wyler, 1988; Chen et al., 2014). In brief, Vero cells (ATCC® CCL-81™, American type culture collection, Manassas, VA) were cultured in 25 cm^2 flasks (Corning®, Corning, NY) using maintenance medium (minimum essential medium (MEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 0.05 mg/ml gentamicin (Life Technologies), 10 units/ml penicillin (Life Technologies), 10 µg/ml streptomycin (Sigma-Aldrich) and 0.25 µg/ml amphotericin (Sigma-Aldrich). Vero cells were inoculated with virus 24 h after reaching 100% confluency. Thereafter, maintenance medium was decanted from contiguous cell monolayers, the monolayer was washed twice with maintenance medium, and the flask was inoculated with 0.5 ml of a mixture of PEDV and post-inoculation medium (MEM supplemented with tryptose phosphate broth (0.3%) (Sigma-Aldrich), yeast extract (0.02%) (Sigma-Aldrich) and trypsin 250 (5 µg/ml) (Sigma-Aldrich). Flasks were then incubated at 37°C with 5% CO_2 for 2 h to allow virus adsorption, after which 5 ml of post-inoculation medium was added to each flask without removing viral inoculum. Flasks were incubated at 37°C with 5% CO_2 until cytopathic effect (CPE) was observed and then subjected to one

freeze-thaw cycle (-80°C). The contents were harvested, centrifuged at 3,000 for 10 min at 4°C to remove cell debris, aliquoted into 2.0 ml microcentrifuge tubes, and stored in -80°C until used.

PEDV titration was performed on confluent Vero cells monolayers grown in 96-well plates (CoStar™, Corning®). Eight 10-fold dilutions of virus stock solution were made using post-inoculation medium. Five wells were inoculated with 100 µl at each dilution, plates were incubated at 37°C with 5% CO_2 for 1 h, and then 100 µl post-inoculation medium was added. Plates were incubated at 37°C with 5% CO_2 for 5 days, after which wells were subjected to IFA staining and evaluated for the presence of virus. Wells with specific staining were classified PEDV-positive. Based on the titration results, the 50% endpoint was calculated as 1 x 10^5 $TCID_{50}$/ml using the Reed-Muench method (Reed and Muench, 1938).

PEDV inoculation

On DPI 0, the PEDV stock solution (1 x 10^5 $TCID_{50}$/ml) was diluted with PBS (1X, pH 7.4, Gibco®, Thermo Fisher Scientific, Grand Island, NY) to an estimated concentration of 1 x 10^3 $TCID_{50}$/ml, mixed 1:4 with milk replacer (Esbilac®, PetAg Inc., Hampshire, IL), and administered orally (5 ml) to all piglets of the PEDV antibody-positive sows, thereby delivering a total PEDV exposure dose of 1 x 10^3 $TCID_{50}$ to each piglet. Thereafter, sows were monitored daily for diarrhea, milking ability, anorexia, and alertness. Piglets were monitored daily for diarrhea, rectal body temperature, dehydration, and ability to stand, walk, and suckle. Piglets unable to suckle, reluctant to stand, or demonstrating ≥ 10% dehydration based on skin tenting were humanely euthanized by intravenous administration of pentobarbital sodium (Fatal-Plus®, Vortech Pharmaceuticals, MI) at a dose of 100 mg/kg.

Biological sample collection

Serum

A total of 210 piglet serum samples and 32 sow serum samples for antibody testing were collected on DPI 0 and DPI 12 (Groups 1 and 3) or 14 (Group 2). Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection system (Becton Dickson, Franklin Lakes, NJ) and serum separation tubes (Kendall, Mansfield, MA). Blood

samples were processed by centrifugation at 1,500 × *g* for 15 min, aliquoted into 2 ml cryogenic tubes (BD Falcon™, Franklin Lakes, NJ), and stored at -20°C until tested.

Mammary secretions A total of 248 colostrum and milk samples for antibody testing were collected from sows between DPI 1 to 12 (Groups 1 and 3) or 14 (Group 2). Sows were administered 20 USP units of oxytocin (VetOne®) to facilitate collection of mammary secretions. Samples were processed by centrifugation at 13,000 × *g* for 15 min at 4°C to remove fat and debris. The defatted samples were then aliquoted into 2 ml cryogenic tubes (BD Falcon™) and stored at -20°C until tested.

Feces

Samples for porcine coronavirus RT-PCR testing included fecal swab samples collected from individual sows immediately prior to receipt of the animals and placed in transport medium (BD BBL™ CultureSwab™ Collection/Transport system, Thermo-Fisher Scientific) and 1204 individual piglet fecal samples collected between DPI 0 and 12. Approximately 1 gram of feces was collected from each piglet using a disposable fecal loop (VetOne®). The sample was mixed with 1 ml PBS (1X pH 7.4, Gibco®) immediately after collection, placed in a 2 ml cryogenic tube (BD Falcon™). Fecal samples of Group 1 and 3 were pooled by litter i.e., 100 µl of feces of each piglet were pooled together in 2 ml cryogenic tube (BD Falcon™) for rRT-PCR testing. Fecal samples of Group 2 were tested individually.

Coronavirus reverse-transcriptase polymerase chain reactions (rRT-PCR)

RNA extraction

In brief, 90 µl of viral RNA was eluted from sow fecal swab samples or 50 µl of piglet fecal:PBS sample (2.5.3 above) using the Ambion® MagMAX™ viral RNA isolation kit (Life Technologies) and a KingFisher® 96 magnetic particle processor (Thermo-Fisher Scientific) following the procedures provided by the manufacturers.

Porcine coronavirus real time RT-PCRs (rRT-PCR)

Sow fecal swab samples and piglet fecal samples were tested for PEDV using a PEDV N gene-based rRT-PCR described by Madson et al. (2014) and performed routinely at the Iowa State University-Veterinary Diagnostic Laboratory (ISU-VDL SOP 9.5263).

Sow fecal swab samples were tested for TGEV using a spike (S) gene-based procedure described by Kim et al. (2007) and performed routinely at the ISU-VDL (ISU-VDL SOP 9.5575). Primers and probes targeting conserved regions of the TGEV S gene were designed to match 9 TGEV strains, including Purdue 46-MAD (GenBank NC00236), TO14 (GenBank AF302264), TS (GenBank DQ201447), SC-Y (GenBank DQ443743), Miller M6 (GenBank DQ811785), TH-98 (GenBank AY676604), HN2002 (GenBank AY587884), 96-1993 (GenBank AF104420), and FS772/70 (GenBank Y00542) (Kim et al., 2007).

Sow fecal swab samples were tested for PDCoV using a membrane (M) gene-based rRT-PCR described by Chen et al. (2014) and performed routinely at the ISU-VDL (ISU-VDL SOP 9.5478). The protocol included positive control standards of known infectivity titers ($TCID_{50}$).

Real time RT-PCR

The eluted RNA, primers, and probe were mixed with commercial reagents (Path-ID® Multiplex One-Step RT-PCR kit, Life Technologies) and the RT-PCR reactions were conducted on an ABI 7500 Fast instrument (Life Technologies) as follows: 48°C for 10 min, 95°C for 10 min, 95°C for 15 s (45 cycles), and 60°C for 45 s. The results were analyzed using an automatic baseline setting with a threshold at 0.1. Quantification cycle (Cq) values < 35 were considered positive for the PDCoV and Cq values < 40 were considered positive for the PEDV and TGEV. Data were reported as "adjusted Cq":

Equation 1.

$$\textit{Adjusted Cq} = \textit{(35 – sample Cq)}$$

Coronavirus antibody assays

PEDV indirect immunofluorescence assay (IFA)

IFA plates were prepared by inoculating confluent monolayers of Vero cells (ATCC® CCL-81™) in 96-well plates (CoStar™, Corning®) with 100 µl/well of PEDV (US/Iowa/18984/2013) at 1 x 10^3 plaque-forming units/ml. The plates were then incubated for 18 to 24 h, after which the inoculum was removed and the cell monolayers fixed with cold acetone:alcohol (70:30) solution (Sigma-Aldrich). Plates were then air-dried, sealed, and stored at -20°C. To perform the test, serum samples were two-fold diluted (1:40 to 1:320) in PBS (1X pH 7.4) and then 100 µl of each dilution was transferred to IFA plates and incubated at 37°C for 1 h. After incubation, the diluted serum samples were removed from test plates, the plates rinsed 3 times with PBS (1X pH 7.4), and 50 µl of 1:50 diluted goat anti-swine IgG antibody conjugated with FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. After a 30 min incubation at 37°C, the plates were rinsed again with PBS (1X pH 7.4) and the cells were observed under an inverted fluorescent microscope for PEDV-specific cytoplasmic staining.

PEDV whole virus antibody ELISA

PEDV (USA/IN/2013/19338E) was used in the PEDV antibody ELISA. In brief, virus was propagated on Vero cells, the flasks subjected to one freeze-thaw, and the harvested material centrifuged at 4,000 × *g* for 15 min to remove cell debris. The virus was then pelleted by ultracentrifugation at 140,992 × *g* for 3 h, after which the virus pellet was washed once with sterile PBS (1X pH 7.4). The purified virus was resuspended in PBS (1X pH 7.4) at a dilution of 1:100 of the original supernatant volume and stored at -80°C. Following titration and optimal dilution, polystyrene 96-well microtitration plates (Nunc, Thermo Fisher Scientific) were manually coated (100 µl/well) with the viral antigen solution and incubated at 4°C overnight. After incubation, plates were washed 5 times, blocked with 300 µl per well of a solution containing 1% bovine serum albumin (Jackson ImmunoResearch Inc., West Grove, PA), and incubated at 25°C for 2 h. Plates were then dried at 37°C for 4 h and stored at 4°C in a sealed bag with desiccant packs. Plate lots with a coefficient of variation ≥10% were rejected.

ELISA conditions for the detection of anti-PEDV IgA and IgG antibodies in serum and colostrum/milk (defatted) specimens, including coating and blocking conditions, reagent concentrations, incubation times, and buffers, were identical. Positive and negative plate controls, i.e., antibody-positive and -negative experimental serum or milk samples, were run in duplicate on each ELISA plate. All samples were diluted 1:50, after which plates were loaded with 100 µl of the diluted sample per well. Plates were incubated at 25°C for 1 h and then washed 5 times with PBS (1X pH 7.4).

To perform the assay, 100 µl of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories Inc., Montgomery, TX) diluted 1:20,000 for serum and colostrum/milk samples or goat anti-pig IgA (Bethyl Laboratories Inc.) diluted 1:3,000 for serum and 1:45,000 for colostrum/milk samples was added to each well and the plates incubated at 25°C for 1 h. After a washing step, the reaction was visualized by adding 100 µl of tetramethylbenzidine-hydrogen peroxide (TMB, Dako North America, Inc., Carpinteria, CA) substrate solution to each well. After a 5 min incubation at room temperature, the reaction was stopped by the addition of 50 µl of stop solution (1 M sulfuric acid) to each well. Reactions were measured as optical density (OD) at 450 nm using an ELISA plate reader (Biotek® Instruments Inc., Winooski, VT) operated with commercial software (GEN5™, Biotek® Instruments Inc.). The antibody response in serum and colostrum/milk samples was represented as sample-to-positive (S/P) ratios calculated as:

Equation 2.

$$S/P\ ratio = \frac{(sample\ OD - blank\ well\ control\ mean\ OD)}{(positive\ control\ mean\ OD - blank\ well\ control\ mean\ OD)}$$

Analysis of the PEDV serum IgG WV ELISA data showed that a S/P cutoff of ≥ 0.40 provided a diagnostic sensitivity of 0.99 (95% CI: 0.96, 1.00) and specificity of 0.98 (95% CI: 0.97, 0.99) (Bjustrom-Kraft et al., 2016). No cutoffs have been established for mammary secretion samples.

PEDV fluorescent focus neutralization (FFN) assay

Colostrum, milk, and serum samples were tested for neutralizing antibody. Prior to FFN testing, milk and colostrum samples were defatted by treatment with Rennet (Rennet from *Mucor miehei*, Sigma-Aldrich). In brief, 5 µl Rennet was added to 1 ml of defatted milk or colostrum and briefly vortexed. The mixture was then incubated at 37°C for 30 min, vortexed, and then centrifuged at 2,000 × *g* for 15 min. The supernatant was then harvested and tested for neutralizing antibody.

To perform the FFN, test samples, antibody-positive control serum, and antibody-negative control serum were heat inactivated at 56°C for 30 min and then 2-fold serially diluted (1:4 to 1:512) in 96-well dilution plates (Axygen®, Corning®) using post-inoculation medium to give a final volume of 100 µl. Then, 75 µl of each dilution was transferred to new dilution plate (Axygen®, Corning®), mixed with 75 µl of PEDV (1 x $10^{3.6}$ $TCID_{50}$/ml) to give final serum dilutions of 1:8 to 1:1024, and incubated at 37°C with 5% CO_2 for 1 h. Vero cell confluent monolayers in 96-well plates (CoStar™, Corning®) were washed twice with post-inoculation medium, inoculated with 100 µl of the sample-virus mixture, incubated at 37°C with 5% CO_2 for 1 h, and washed twice. 100 µl of post-inoculation medium was then added to each well and the plates incubated at 37°C with 5% CO_2 for 48 h. Finally, cells were fixed with 80% cold acetone:alcohol (80:20), stained with FITC-conjugated monoclonal antibody (SD6-29, Medgene Labs, Brooking, SD) for 1 h, and observed under an inverted fluorescent microscope for PEDV-specific cytoplasmic staining. Positive neutralizing endpoints were determined as the highest dilution resulting in a ≥ 90% visual reduction in fluorescing foci relative to the antibody-negative serum control. Plates in which the positive control deviated more than 2-fold from its expected antibody titer were considered invalid.

Data analysis

Area under the curve (AUC) analysis was used to calculate total PEDV mammary secretion antibody for each sow using FFN, WV-ELISA IgA, and WV ELISA IgG testing results for DPI 1 through 12 (MedCalc® version 16.4.3, Ostend, Belgium). Sow 5 was not included in this analysis because all piglets succumbed to PEDV on DPI 5 and mammary secretions were

not collected thereafter. Thereafter, the association between DPI -7 serum antibody levels and total PEDV mammary secretion antibody were analyzed by regression analysis (SAS® 9.4, SAS® Institute Inc., Cary NC, USA).

Means and standard errors (SE) for percent body weight change, body temperature, PEDV fecal shedding (adjusted Cq), FFN antibody titer, WV-ELISA IgA and IgG S/P were calculated for each day using SAS® 9.4 (SAS® Institute Inc., Cary NC, USA). For individual piglets, the percent change in body weight was calculated relative to the weight of the piglet at 1 day of age:

Equation 3.

$$Percent\ change = \frac{(weight - weight\ at\ 1\ day\ of\ age)}{(weight\ at\ 1\ day\ of\ age)} \times 100$$

Prior to analysis, FFN data were transformed by dividing the reciprocal by 10 and taking the $\log_2$. Results were back-transformed and expressed as antibody titers. One-way analysis of variance (ANOVA) with repeated measures (DPI) was used to assess differences in body weight change, body temperature, PEDV fecal shedding (adjusted Cq), and antibody responses (FFN, IgA, and IgG) among groups over time. When differences were detected, compound symmetry covariance parameter estimates were used in the repeated measures analysis to detect differences among groups by DPI. Differences among groups in the time to death were analyzed using proportional hazard regression analysis with a robust sandwich covariance matrix estimate.

Results

Sows

All sows were clinically normal throughout the study. Sows exhibited normal maternal behavior and litters were kept intact and with their dam throughout the study. All sow fecal samples collected prior to inoculation on DPI 0 were PEDV, TGEV, and PDCoV qRT-PCR-negative.

Group 1 (n = 6 sows) and Group 3 (n = 2) consisted of animals without prior exposure to PEDV. All serum samples collected at DPI -7 were negative by FFN (antibody titer < 1:8) and PEDV WV IgG ELISA (S/P < 0.4). All Group 1 serum samples collected on DPI 14 were negative by FFN and positive on the WV IgG ELISA (mean S/P 1.8, SE 0.4). In Group 3, serum samples collected at DPI 14 were negative by both FFN (< 1:8) and PEDV WV IgG ELISA (S/P < 0.4).

Group 2 (n = 8 sows) consisted of animals previously exposed to PEDV. All serum samples collected at DPI -4 were positive by FFN (mean antibody titer 1:8, SE 0) and PEDV WV IgG ELISA (mean S/P 1.6, SE 0.2). Serum samples collected at 12 DPI showed a strong response to PEDV exposure compatible with an anamnestic response, i.e., FFN (mean antibody titer 1:26, SE 1) and WV IgG ELISA (mean S/P 2.2, SE 0.2).

As shown in Table 1, total mammary secretion antibody levels were relatively uniform among sows within Groups 1 and 2 (samples were not collected from Group 3). Day-by-day comparisons showed higher antibody levels (FFN, IgA, IgG) in Group 2 (ANOVA, $p < 0.0001$) at all sampling points for all tests, with the exception of the WV IgG ELISA on DPIs ≥ 4 (Fig. 1). Correlation analysis did not detect an association between Group 2 sow serum antibody levels (FFN, IgG) on DPI -4 and FFN, IgG, or IgA levels in mammary secretion samples collected on DPI 0 ($p > 0.05$).

Piglets

All piglets were clinically normal in appearance and behavior prior to PEDV inoculation. As shown in Table 2, testing of serum samples collected on DPI 0 showed that Group 1 and 3 piglets were antibody negative by FFN, PEDV WV IgA ELISA, and PEDV WV IgG ELISA. In contrast, Group 2 piglets were positive on all three assays due to maternal antibody. All piglet fecal samples collected prior to inoculation on DPI 0 were PEDV qRT-PCR-negative.

Piglets were 4 days of age (Group 1) or 2 days of age (Group 2) at the time of PEDV inoculation. Diarrhea was observed in 1 of 11 Group 1 piglets on DPI 1, increasing to 6 of 8

piglets on DPI 4 and declining thereafter such that no diarrheic piglets were observed in the group on DPI 7. In Group 2, diarrhea was observed in 2 of 91 piglets on DPI 1. This increased to 26 of 87 piglets on DPI 4 and decreased thereafter. On DPI 11, 1 diarrheic piglet was observed among the 70 pigs remaining in the group. No diarrhea was observed in Group 3 piglets (uninoculated controls) at any time in the study.

PEDV RT-PCR positive fecal samples were detected in Groups 1 and 2 from DPI 1 through 12 (Fig. 2A). Compared to Group 1, Group 2 piglets shed significantly less virus on DPIs 1 to 5 (ANOVA, $p < 0.05$). Thereafter, no significant difference was found between the two groups in the level of fecal viral shedding, although numerically lower adjusted Cqs were suggestive of less virus shedding in Group 2.

Body temperature data collected on DPIs -1 and 0 was used to calculate mean piglet body temperature (38.9°C) and 95% confidence intervals (38.3°C, 39.1°C). Using these values as the "normal range", the mean body temperature of Group 1 piglets was below the lower bound of the normal range on DPIs 2 through 8 and again on DPI 10 (Fig. 2B). The mean body temperature of Group 2 piglets was within the normal range throughout the study. A significant difference in body temperature was detected between Group 1 and 2 piglets at all sampling points (ANOVA, $p < 0.05$) except DPIs 9, 11, and 12. Within Group 2, repeated measures ANOVA analysis detected a significant difference in body temperature response ($p < 0.05$) among litters, but this could not be explained by differences in mammary secretion antibody levels among Group 2 sows.

The piglet rate of growth was expressed as percent weight change relative to piglet weight on the first day post farrowing. Statistical analysis found differences in the rate of growth between Group 1 and 2 piglets at all sampling points (ANOVA, $p < 0.05$) except DPIs 2 to 4 (Fig. 2C). Thus, the two groups were different from the beginning of the observation period, with a significantly lower rate of growth in Group 2 piglets. After DPI 5 and through the end of the trial, Group 2 piglets performed better than Group 1 piglets ($p < 0.05$). Further analysis based on repeated measures ANOVA found no significant difference in percent weight change among litters within Group 2 ($p > 0.05$).

All uninoculated control piglets (Group 3, n = 22) survived to the end of the observation period. In contrast, 10 piglets (91%) in Group 1 died between DPIs 4 and 10 and 14 piglets (15%) in Group 2 died between DPIs 1 and 9 (Fig. 2D). Among these piglets, 79% (n = 11) were humanely euthanized because they met welfare thresholds (unable to suckle, reluctant to stand, or ≥ 10% dehydration). Proportional hazard regression analysis comparing Groups 1 and 2 found a significant difference in piglet survivability ($p < 0.0001$). Proportional hazard regression comparing litters within Group 2 found no difference in the rate of survival ($p > 0.05$).

Discussion

Neonatal pigs are born agammaglobulinemic, possess limited peripheral lymphoid cells, undeveloped lymphoid tissues, and no effector and memory T-lymphocytes (Sinkora and Butler, 2009). To provide some level of immunological protection, neonatal piglets receive maternal cell-mediated (CMI) and humoral immune components through the ingestion of colostrum and milk (Saif and Jackwood, 1990). In this discussion, we review and discuss the elements of this process in the context of neonatal protection against PEDV and other coronaviruses.

Sow mammary secretions contain a significant number of immune cells (~1×10^7 per ml), the majority of which are neutrophils (71.7%) and lymphocytes (26.4%), with fewer macrophages (1.3%), epithelial cells (0.4%), and eosinophils (0.2%) (Evans et al., 1982). Cell numbers and types reflect both the developmental stage of the mammary gland and the physiological and/or immunological conditions of the sow (Magnusson et al., 1991). After ingestion by piglets, lymphocytes are able to cross the duodenal and jejunal epithelium by intercellular migration for at least one week after birth (Bianchi et al., 1999; Tuboly et al., 1988; Williams, 1993). For reasons that are not clear, only maternally-derived cells have this capacity (Tuboly et al., 1988; Williams, 1993). That is, cross-fostered piglets cannot absorb cells from a substitute dam (Bandrick et al., 2011). Once absorbed, these cells migrate to mesenteric lymph nodes and a variety of other tissues, including liver, lung, spleen, and the

lamina propria and submucosal spaces of the duodenum and jejunum (Tuboly et al., 1988; Tuboly and Bernath, 2002; Williams, 1993). These cells retain functionality within the neonate. Thus, peripheral blood mononuclear T-lymphocytes and peripheral B-lymphocytes in piglets that received colostral cells showed higher responses to mitogens, e.g., phytohemagglutinin and concavalin A, compared to controls (Williams, 1993). Research on the role of colostral immune cells in protecting piglets against coronaviruses is scant, but the transfer of peripheral blood mononuclear cells from adult pigs into neonates was shown to delay the onset of TGEV enteritis (Cepica and Derbyshire, 1984).

In addition to maternal CMI, antibody provided in colostrum and milk protects the piglet in the interval between birth and the development of a functional immune system. Curtis and Bourne (1971) reported that sow colostrum contained an average of 61.8 mg/ml IgG1, 40.3 mg/ml IgG2, 9.7 mg/ml IgA, and 3.2 mg/ml IgM. At 72 hours post-farrowing, levels had declined to 1.9 mg/ml IgG, 1.3 mg/ml IgG2, 3.4 mg/ml IgA, and 1.2 mg/ml of IgM. Bourne and Curtis (1973) showed that nearly 100% of IgG, 40% of IgA, and 85% of IgM in colostrum was derived from serum; whereas, 70% of IgG, and ≥ 90% of IgA and IgM in milk was produced locally in the mammary glands.

In late pregnancy, lactogenic immunity predominantly involves IgG. Coinciding with high progesterone and low estrogen levels, binding of the IgG1 heavy chain by Fcγ receptors on mammary epithelial cells promotes the selective transport of IgG from serum into mammary secretions (Butler, 1998; Chamley et al., 1973; Hammer and Mossmann, 1978; Killian et al., 1973; Porter, 1988; Schnulle and Hurley, 2003; Watson, 1980). Using a passive antibody model, Poonsuk et al. (2016) found that PEDV-inoculated neonates with circulating anti-PEDV antibodies returned to normal body temperature earlier and exhibited higher survivability compared to piglets without PEDV antibody. Although circulating antibody did not neutralize the negative effects of PEDV on growth nor reduce shedding of PEDV in feces, the results implied that colostral IgG contributed to the protection of neonates against PEDV.

Postpartum, lower serum progesterone and higher serum corticoid and prolactin levels induce milk production and suppress colostrum production (Banchero et al., 2006; Delouis, 1978; Hartmann et al., 1984). This switch coincides with lower IgG and higher IgA concentrations in mammary secretions (Ash and Heap, 1975; Devillers et al., 2004; Foisnet et al., 2010). After parturition, lactogenic immunity predominantly involves secretory IgA (S-IgA) produced from plasma cells in mammary gland tissues. Beginning shortly before parturition and continuing throughout lactation, IgA plasma cells preferentially migrate from mesenteric lymph nodes to the mammary glands (Roux et al., 1977). Mammary gland lymphocyte accumulation differs between gilts and sow, i.e. multiparous sows have more mammary tissue and are able to accumulate more plasma cells than primiparous females (Bischof et al., 1994; Sordillo et al., 1997; Sordillo and Streicher, 2002). Therefore, sows produce higher concentrations of S-IgA in mammary secretions (Cabrera et al., 2012; Klobasa et al., 1986). Like S-IgA, secretory IgM (S-IgM) is produced from IgM plasma cells in mammary gland tissues (Sordillo et al., 1997), but the mechanisms that regulates S-IgM plasma cell localization postpartum are not clear.

Lactogenic immunity against coronaviruses requires "productive" enteric viral replication to stimulate the development of specific IgA plasmablasts (Langel et al., 2016). Thus, parenteral immunization of sows with modified-live TGEV vaccines increased IgG in colostrum, but not IgA (Saif and Jackwood, 1990, Bohl et al., 1972a; Saif et al., 1972). Following enteric infection, IgA plasmablasts migrate to the mammary glands where they contribute S-IgA to milk and colostrum (Bohl et al., 1972a). Bohl et al. (1972b) found that sows orally inoculated with virulent TGEV produced high titers of S-IgA in mammary secretions and protected their piglets against the clinical effects of TGEV, whereas highly attenuated oral TGEV vaccines resulted in lower S-IgA antibody titers in mammary secretions and inadequate lactogenic immunity in suckling piglets (Jackwood et al., 1995; Moxley and Olson, 1989; Saif, 1999a,b).

Within the mammary gland, polymeric immunoglobulins (dimeric S-IgA or pentameric S-IgM) secreted by local plasma cells bind to polymeric immunoglobulin receptors (pIgR) on the basolateral pole of the secretory epithelial cell (Johansen et al., 2000) and are then

transported through the endosomal compartment to the luminal surface of the epithelial cell (Rojas and Apodaca, 2002). Upon reaching the luminal side of the epithelial cell, the pIgR molecule is enzymatically cleaved; a process that leaves a receptor fragment ("secretory component") attached to the immunoglobulin molecule (Hunziker and Kraehenbuhl, 1998;). The secretory component functions to protect the hinge region of S-IgA and S-IgM from protease cleavage, thereby extending their stability on mucosal surfaces (Hurley and Theil, 2011). The concentration of S-IgA and S-IgM in milk is dependent upon the rate of transportation across epithelial cells, but the rate of transport is dependent upon the expression of pIgR on mammary epithelial cells, which is under the control of hormones responsible for the initiation of lactation (Rosato et al., 1995; Scicchitano et al., 1986; Sheldrake et al., 1984). In sows, the hormonal regulation of the expression of pIgR on mammary epithelial cells has not been described, although it is known that pIgR density is highest from 0 to 3 days postpartum (Schnulle and Hurley, 2003).

In addition to endogenous hormones, exogenous hormones also affect colostrum yield and composition. In ewes, induction of parturition using estradiol benzoate or dexamethasone m-sulfobenzoate reduced IgG1, IgG2, IgM, and IgA concentrations in colostrum (Dawe et al., 1982). In cows, Field et al. (1989) showed that synchronization using dexamethasone m-sulfobenzoate reduced the total IgG in colostrum by ~43%. Further, Field et al. (1989) found that induction with prostaglandin F2 alpha resulted both in a reduction of colostrum production and a lower concentration (44% decrease) of IgG in colostrum. Similar to other species, sows chemically induced to farrow trended toward the production of less colostrum and that the colostrum contained less fat (Farmer and Quesnel, 2009; Jackson et al., 1995; Milon et al., 1983). This is relevant because the sows in this study were induced to farrow using dinoprost tromethamine (Lutalyse®), a tromethamine salt of prostaglandin F2 alpha, on day 113 of gestation. It is possible that this procedure may have impacted the concentration and total quantity of colostral antibody. Although the results of this study showed that induction of sows did not affect the overall capacity of lactogenic humoral immunity to protect piglets against PEDV, this may be a consideration in the field when optimization of herd immunity against PEDV is an objective.

Within the neonatal digestive tract, immunoglobins remain intact because proteolytic activity in the digestive tract is low and is further reduced by "sow colostrum trypsin inhibitor (SCTI)" (Jensen, 1978). Trypsin is able to cleave immunoglobulins, but SCTI irreversibly binds trypsin, thereby protecting proteins in mammary secretions (Chamberlain et al., 1965; Kunitz, 1947). The small intestine of the neonate is lined with mucosal epithelial cells capable of non-selectively absorbing any intact macromolecules, including immunoglobulins (Hurley and Thiel, 2011). Immunoglobulins in the lumen of the intestine are first non-selectively taken up by enterocytes via pinocytosis (Bush and Staley, 1980). The pinocytosed immunoglobulins are released in the lamina propria and then diffuse to the intestinal lymphatic system, from where they enter the circulatory system (Moog, 1979; Murata and Namioka, 1977). The neonatal intestinal epithelium retains the ability to pinocytose macromolecules for a limited period of time. Murata and Namioka (1977) reported that non-specific pinocytosis of colostral immunoglobulins disappeared by 2 hours after birth in the duodenum, 48 hours in jejunum, and 72 hours in the ileum ("gut closure"). Within 9 days of birth, the mucosal epithelium is replaced with new intestinal epithelial cells (Moog, 1979; Smith and Jarvis, 1978). These cells are able to pinocytose macromolecules, but cannot transfer immunoglobulins to the lamina propria (Rooke and Bland, 2002).

Absorbed maternal IgM, IgA, and IgG undergo transudation through the epithelial cells to reach the intestinal mucosal surfaces, where they play a major role in protecting neonates against enteric pathogens, e.g., TGEV and rotavirus (Bohl et al., 1972b; Saif et al., 1972; Saif, 1999a; Ward et al., 1996). Colostral IgG appears in the neonatal circulatory system within 48 hours after birth (Curtis and Bourne, 1973; Porter, 1988; Watson, 1980). Depending on the sow's antibody profile, circulating maternal IgG can ameliorate the clinical effects of systemic infections, e.g., porcine parvovirus, porcine circovirus, and (Langel et al., 2016; Ostanello et al., 2005).

After gut closure, the majority of macromolecules are degraded by digestive enzymes, but some immunoglobulins remain intact and are transported across intestinal epithelium via antibody-specific FcRn receptors on gut epithelial cells (Stirling et al., 2005). By this mechanism, IgG, IgM, and IgA in mammary secretions may undergo selective transcytosis

into enterocytes. In addition, S-IgA and S-IgM are stabilized by binding to secretory component and can be transcytosed by Fc receptor of enterocytes without degradation (Brandtzaeg, 1981).

The objective of the experiment was to quantify the protective effects of lactogenic immunity by comparing the course of PEDV infection in neonatal piglets farrowed by immune vs naïve dams. Overall, piglets that received lactogenic antibody shed less PEDV and exhibited better thermoregulation, higher growth rates, and higher survivability. These results are compatible with observations for other swine coronaviruses. Bohl et al. (1972) found that sows previously infected with TGEV delivered levels of lactogenic immunity that reduced TGEV shedding, morbidity, and mortality in their piglets. Wesley and Lager (2003) reported that TGEV inoculated sows provided strong lactogenic antibody responses resulted in effectively protection against TGEV in neonates on basis of reduced clinical illness, and increased litter survival rates (Wesley and Lager, 2003).

Notably, significant differences were found among litters from PEDV-immune dams in the quantity of virus shedding ($p < 0.05$) and return to normal body temperature ($p < 0.05$), but not in piglet survival rate. Although these differences were detected, it was not possible to assign a causal role to antibody isotype or concentration in mammary secretions because of sample size. In addition, some potentially contributing factors were not accounted for, e.g., the amount of colostrum or milk consumed by each pig, and other remain unknown, e.g., the role of CMI. Given that the ultimate goal of this line of research is to provide specific guidelines for monitoring and maintaining lactogenic immunity against PEDV infection in sow herds, these remain important topics for future research.

Declaration of conflicting interests

The author(s) declare that there are no conflicts of interest with respect to their authorship and/or the publication of this manuscript.

Funding

This work was supported in part by Pork Checkoff funds distributed through the National Pork Board (#09-193), Zoetis, and IDEXX Laboratories, Inc.

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Tables

Table 1. Total PEDV mammary secretion antibody by group and sow based on area under the curve (AUC) analysis*

		FFN		IgA ELISA		IgG ELISA	
Group	Sow	AUC	Maximum FFN titer	AUC	Maximum IgA S/P	AUC	Maximum IgG S/P
1**	1	11.3	1:4	5.2	1.2	1.7	0.9
	2	12.5	1:16	2.7	0.2	1.2	0.6
	3	9.0	1:8	3.1	0.5	4.2	1.2
	4	12.5	1:4	2.9	0.6	1.9	0.7
	6	13.2	1:16	5.8	2.0	1.3	0.9
	$\bar{x}$ (SE)	11.7 (0.7)	1:8 (0.4)	3.9 (0.7)	0.9 (0.3)	2.1 (0.5)	0.9 (0.1)
2	7	51.0	1:256	24.7	2.3	13.3	3.0
	8	76.6	1:1024	23.7	2.1	15.1	3.3
	9	47.3	1:64	21.2	2.6	13.1	1.9
	10	75.1	1:512	24.1	2.2	14.1	2.3
	11	68.6	1:256	33.2	2.7	28.8	3.5
	12	68.5	1:128	26.8	2.4	19.4	2.9
	13	80.0	1:256	32.6	2.7	23.2	4.4
	14	41.0	1:64	26.4	1.7	16.3	3.3
	$\bar{x}$ (SE)	63.5 (5.3)	1:223 (0.5)	26.6 (1.5)	2.3 (0.1)	18.0 (2.0)	3.1 (0.3)

* Area under the curve (AUC) for each sow and assay was calculated using mammary secretion antibody data for samples collected DPI -1 to 12 (MedCalc® version 16.4.3, Ostend, Belgium).

** Sow 5 not included in this analysis because all piglets succumbed to PEDV on DPI 5 and mammary secretions were not collected thereafter.

Table 2. Piglet serum antibody levels by group and day post inoculation (DPI)

Assay	Group	DPI 0	No. of piglets	DPI 12 or 14*	No. of piglets
FFN antibody titer arithmetic mean (SE)	1	< 1:8 (0)	11	1:16	1
	2	1:39 (1.1)	91	1:13 (1.1)	78
	3	< 1:8 (0)	22	< 1:8 (0)	22
PEDV IgA ELISA least square mean S/P (SE)	1	0.6 (0.1)	11	1.7	1
	2	2.5 (0.06)	91	1.9 (0.07)	78
	3	0.6 (0.01)	22	0.3 (0.009)	22
PEDV IgG ELISA least square mean S/P (SE)	1	0.2 (0.05)	11	2.1	1
	2	5.4 (0.06)	91	2.1 (0.1)	78
	3	0.3 (0.01)	22	0.02 (0.004)	22

* Observation period ended on DPI 12 for Groups 1 and 3 and DPI 14 for Group 2.

Figures

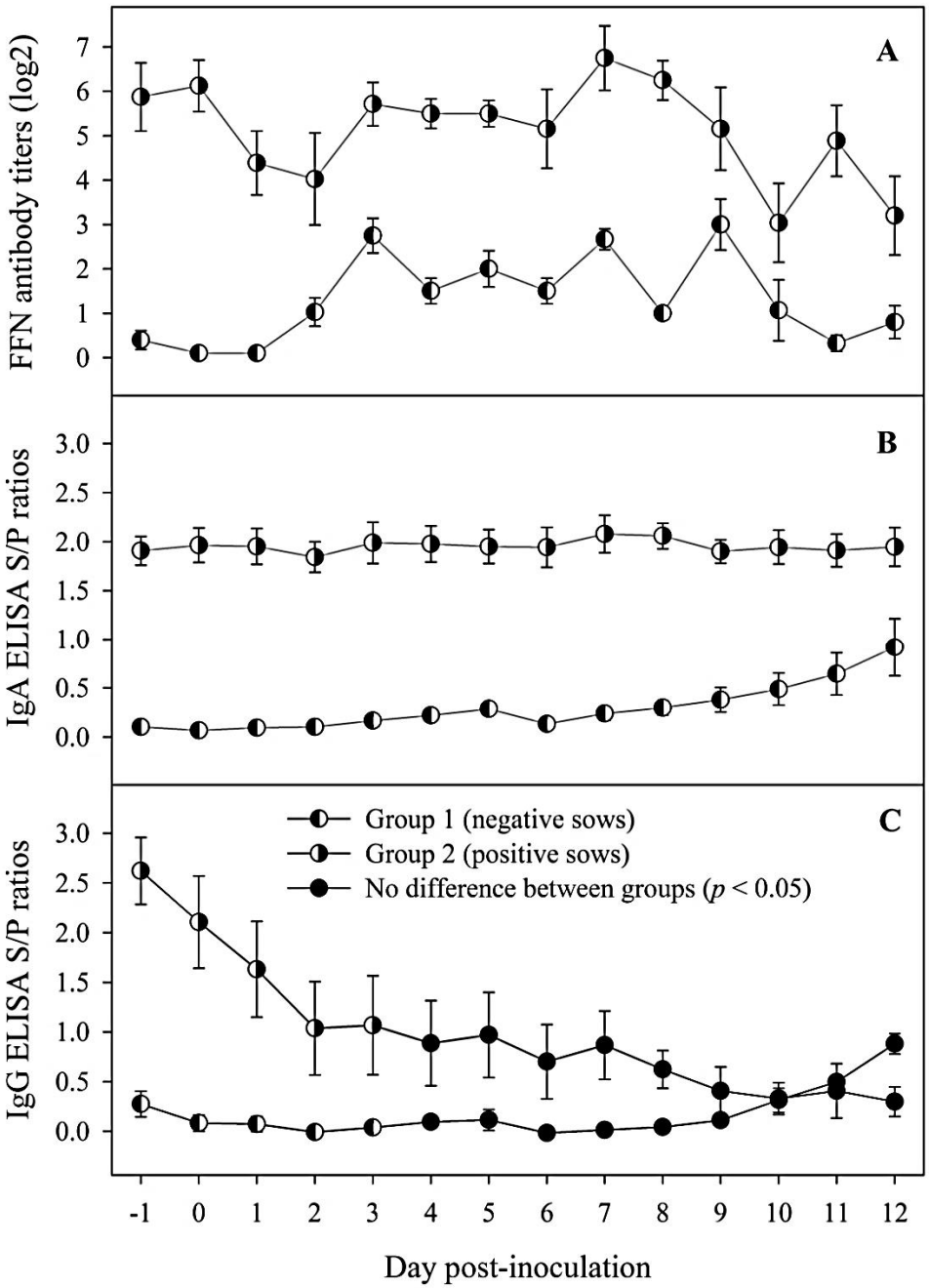

Figure 1. PEDV antibody in mammary secretions tested by (A) fluorescent focus neutralization (FFN) assay, (B) PEDV indirect IgA ELISA, and (C) PEDV indirect IgG ELISA. Group 1 sows (n = 6) were PEDV serum antibody negative and Group 2 sows (n = 8) were PEDV serum antibody positive. Piglets in both groups were orally inoculated with PEDV (USA/IN/2013/19338E) on DPI 0.

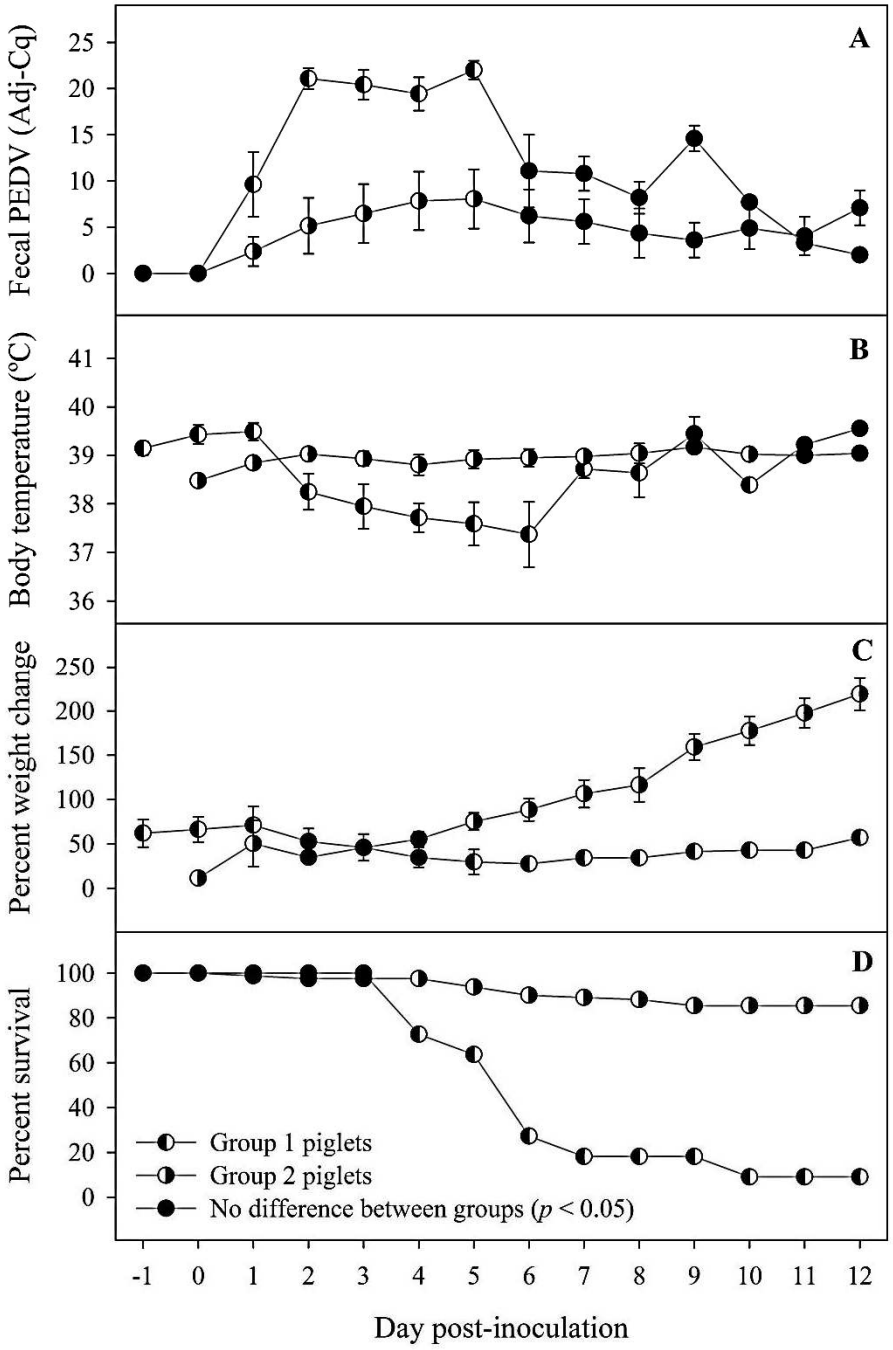

Figure 2. Clinical data from piglets orally inoculated with PEDV (USA/IN/2013/19338E): (A) PEDV fecal shedding (adjusted Cq = 35 - sample Cq); (B) mean body temperature (°C); (C) percent weight change; and (D) percent survival within the group. Group 1 piglets (n = 11) were from serum antibody negative sows and Group 2 piglets (n = 91) were from serum antibody positive sows.

CHAPTER 5. THE EFFECT OF CHEMICAL CLARIFICATION OF ORAL FLUIDS ON PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) ISOTYPE-SPECIFIC (IgG, IgA) ELISA RESPONSES

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Modified from a manuscript to be submitted to BMC Veterinary Research

ABSTRACT

Background: Porcine epidemic diarrhea virus (PEDV) became endemic in the western hemisphere following its introduction in 2013. Therefore, swine producers and veterinarians need an efficient method to routinely monitor herd immunity in order to know when to take appropriate preventative actions. Monitoring oral fluid samples for IgG and/or IgA PEDV antibody is one method to achieve this goal. However, oral fluids contain particles of feed, feces, and inorganic material from the environment which, in the severest cases, may affect test performance. Clarification of oral fluids by centrifugation or filtration is not practical in high through-put diagnostic laboratories because they add additional time and expense. In this study, swine oral fluid samples were clarified using lyophilized chitosan-based formulas (A, B, C) and then tested by PEDV IgG and IgA ELISAs. To evaluate both the immediate and residual effects of treatment on antibody detection, samples were tested immediately post-treatment, then stored at 4°C and re-tested on day post-treatment (DPT) 2, 4, and 6.

Results: Formulations were easily and rapidly rehydrated by the addition of oral fluid and chitosan was shown to effectively clarify samples. Statistical analysis comparing treated to untreated oral fluid samples on DPT 0 found that neither chitosan nor Tween-20® affected

the oral fluid ELISA IgA and IgG S/P results ($p > 0.05$). Furthermore, pairwise comparisons of DPT 0 to DPT 2, 4, and 6 results detected no significant differences ($p > 0.05$) in IgA and IgG S/P ratios, i.e., treated oral fluid samples were stable over time.

Conclusion: Chitosan efficiently clarified oral fluid specimens without affecting the results of the PEDV IgG and IgA antibody ELISAs. From a larger perspective, given the large number of coagulants available, this study should be interpreted as the initiation of a new line of research in oral fluid diagnostics.

KEYWORDS (3 -6)

Porcine epidemic diarrhea virus, oral fluid, antibody, clarification

Introduction

Porcine epidemic diarrhea virus (PEDV), a member of family *Coronaviridae*, is a single-stranded positive sense RNA virus and an important cause of enteric disease in swine. The virus is highly contagious and produces significant morbidity and mortality in neonatal pigs (Song and Park, 2012; Thomas et al.,2015). The virus was first reported in 1971 in England, but North Americ remained free until 2013 (Huang et al., 2013; Pensaert and De Bouck, 1978; Stevenson et al., 2013). Following its introduction, the virus spread rapidly in the Western Hemisphere and it is now endemic in the region.

Swine producers and veterinarians need a practical and efficient method to monitor population immunity in order to know when to take preventive action. Humoral immunity is key to the prevention of clinical outbreaks and a variety of PEDV antibody assays have been developed for use with a variety of specimens, e.g., serum (Bjustrom-Kraft et al., 2016; Gerber et al., 2014; Okda et al., 2015), oral fluid (Bjustrom-Kraft et al., 2016), feces (Gerber and Opriessnig, 2015), and mammary secretions (Gerber et al., 2014; Poonsuk et al., 2016a; Poonsuk et al., 2016b; Song et al., 2016). In the majority of cases, the focus has been on testing individual animal serum samples, but aggregate specimens, such as oral fluids, offer specific advantages and have come into common use for a variety of endemic pathogens, including PEDV (Ramirez et al., 2012). Bjustrom-Kraft et al. (2016) found that both PEDV

IgG and IgA antibody were detectable in oral fluid samples by 13 days post-exposure, but the IgA response was more robust and of longer duration than the IgG response. Notably, the oral fluid IgA ELISA sample-to-positive (S/P) ratios continued to increase for ~90 days post exposure.

In diagnostic medicine, it is recognized that some specimens are improved by processing or treating prior to testing. For example, centrifugation of colostrum and milk samples (Bohl et al., 1972; Rainard, 2010) or incubation of these samples with chymosin or rennet (Rainard, 2010) improved antigen-antibody binding in ELISA assays by separating lipid from serum components. Gerritsen et al. (1991) found that the addition of EDTA (0.1 M, 20% v/v) to urine specimens prevented DNAse activity during storage and transport of the samples. Similarly, fecal samples may require centrifugation, chemical treatment, and/or filtration prior to testing (Cavallini et al., 2000; Garcia et al., 1983; Garcia et al., 1987; Riepenhoff-Talty et al., 1981). Unlike these examples, swine oral fluid does not inherently contain components that affect antibody-based testing, i.e., processing prior to testing is not mandatory. However, samples collected in the field routinely contain fine particulates, i.e., feces, soil, and feed particles, that potentially impact pipetting accuracy and/or test performance. Particularly as laboratories move to higher-throughput technologies, e.g., robotics, it may be desirable to standardize the condition of oral fluid samples.

The options available for removal of particulates from oral fluids include centrifugation, filtration, or precipitation of suspended particulates through chemical treatments. Filtration and centrifugation are not practical in high-throughput diagnostic laboratories because they add considerable expense and time to the testing process. In brewing and winemaking, "clarification" is the process of removing insoluble particles from the product. Applying that approach to oral fluids, the objective of this study was to evaluate clarification of swine oral fluid samples using a chemical coagulant. Given the diagnostic function of the sample, key outcomes of interest included the effect of the treatment on the diagnostic performance of PEDV IgG and IgA ELISAs. To fully explore the residual effect of the treatment on antibody detection, the study also evaluated the stability of PEDV-specific IgG and IgA in samples held at 4°C over the course of one week.

Materials and Methods

Experimental design

The objective of this study was to evaluate the effect of chemical clarification of oral fluid specimens on PEDV antibody ELISA (IgG and IgA) responses over time. Three chemical treatments were evaluated using oral fluid samples collected under experimental conditions (Study One) and under field conditions (Study Two). In Study One, 7-week-old PEDV-negative pigs (n = 16) were randomly assigned to negative control (n = 6 pigs housed 2 pigs per pen) or PEDV-inoculated (n = 10 pigs housed 2 pigs per pen) treatment groups. Serum samples were collected from all pigs on day post inoculations (DPIs) -7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42. Pen oral fluid samples were collected on DPIs -3, 0, 5, 10, 15, 20, 25, 30, 35, and 42. In Study Two, 10-week-old PEDV-negative gilts (n = 20) housed in one pen were orally inoculated with PEDV-positive feces collected from clinically affected pigs in a commercial production system. Serum samples were collected from all pigs on DPIs -4, 0, 7, 14, 21, and 28. Pen oral fluid samples were collected from the pen on DPIs -4, 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, and 42.

Oral fluid samples (n = 104) collected in Studies One and Two were subdivided into 4 aliquots. Each aliquot was subjected to one of 3 chemical treatments, with the fourth aliquot serving as a non-treatment control. All aliquots were tested by PEDV IgG and IgA ELISAs (day 0), then kept at 4°C and tested again on day post treatment (DPT) 2, 4, and 6. Likewise, serum samples were tested by PEDV IgG and IgA ELISAs (day 0), stored at 4°C, and tested again on 2, 4, and 6. The effect of treatments and storage time on PEDV ELISA sample-to-positive (S/P) results were analyzed by 2-way ANOVA and Bland-Altman analyses.

Animal housing and care

Studies One and Two were conducted under the approval of the Iowa State University Office for Responsible Research. In Study One, 16 7-week-old pigs acquired from one commercial swine farm were housed in the Iowa State University Livestock Infectious Disease Isolation Facility (LIDIF). Pens were equipped with nipple drinkers and pigs were fed an antibiotic-

free commercial diet twice daily (Heartland CO-OP, West Des Moines, IA, USA). To verify their negative status for PEDV and transmissible gastroenteritis virus infections, serum samples collected 2 weeks prior shipping and serum and fecal swabs collected on DPI -10 were tested by pathogen-specific ELISAs and real-time reverse transcriptase-polymerase chain reaction (rRT-PCR). Pigs were randomly assigned to one of 2 groups, i.e., negative control group (n = 6) and PEDV-inoculated group (n = 10). Throughout the experiment, pigs were closely observed twice daily by researchers, animal caretakers, and veterinary staff. All personnel involved in the experiment had received institutional approval for working with swine and conducting the necessary procedures.

In Study Two, 20 9-week-old pigs were acquired from one commercial swine farm known to be free of PEDV infection on the basis of routine monitoring. The pigs were housed in one pen in a commercial production facility equipped with nipple drinkers, and concrete slatted flooring. Animals were fed a commercial, antibiotic-free corn/soy swine diet (Nutra Blend LLC., Neosho, MO) at a rate of 5 pound per animal per day. To verify their PEDV negative status, serum and oral fluid specimens were collected from all pigs on DPI -4 and tested by PEDV ELISA and rRT-PCR. The pigs were observed once daily by animal caretakers throughout the experiment.

PEDV propagation and titration

In Study One, PEDV isolate USA/IN/2013/19338E (Chen et al., 2014) was propagated on Vero cells (African green monkey kidney) using methods described elsewhere (Hofmann and Wyler, 1988). In brief, Vero cells (ATCC® CCL-81™, American type culture collection, Manassas, VA) were cultured in 25 cm^2 flasks (Corning®, Corning, NY) using maintenance medium (minimum essential medium (MEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 0.05 mg/ml gentamicin (Life Technologies), 10 units/ml penicillin (Life Technologies), 10 µg/ml streptomycin (Sigma-Aldrich) and 0.25 µg/ml amphotericin (Sigma-Aldrich). Vero cells were inoculated with virus 24 h after reaching 100% confluency. Briefly, maintenance medium was decanted from contiguous cell monolayers, the monolayer was washed twice with post-inoculation medium [MEM supplemented with

tryptose phosphate broth (0.3%) (Sigma-Aldrich), yeast extract (0.02%) (Sigma-Aldrich) and trypsin 250 (5 µg/ml) (Sigma-Aldrich)], and the flask was inoculated with 0.5 ml of a mixture of PEDV and post-inoculation medium. Flasks were then incubated at 37°C with 5% CO_2 for 2 h to allow virus adsorption, after which 5 ml of post-inoculation medium was added to each flask without removing viral inoculum. Flasks were incubated at 37°C with 5% CO_2 until cytopathic effect (CPE) was observed in 80% of the cells and then subjected to one freeze-thaw cycle (-80°C). The contents were harvested, centrifuged at 3,000 × *g* for 10 min at 4°C to remove cell debris, aliquoted into 2.0 ml microcentrifuge tubes, and stored at -80°C.

PEDV titration was performed on confluent Vero cell monolayers grown in 96-well plates (CoStar™, Corning®). Eight 10-fold dilutions of virus stock solution were made using post-inoculation medium. Five wells were inoculated with 100 µl at each dilution, plates were incubated at 37°C with 5% CO_2 for 1 h, and then 100 µl of post-inoculation medium was added. Plates were incubated at 37°C with 5% CO_2 for 5 days, after which wells were subjected to staining using a FITC-conjugated monoclonal antibody SD6-29 (Medgene Labs, Brookings, SD) against the PEDV nucleocapsid protein and evaluated for the presence of virus. Wells with specific staining were classified as PEDV-positive. Based on the titration results, the 50% endpoint was calculated as 1 x 10^6 $TCID_{50}$/ml using the Reed-Muench method (Reed and Muench, 1938).

PEDV inoculation

In Study One, pigs were inoculated with the 8th cell culture passage of PEDV USA/IN/2013/19338E by mixing virus stock solution (15 ml of 1 x 10^6 $TCID_{50}$/ml) with 5 ml milk replacer (Esbilac®, PetAg Inc., Hampshire, IL). The final volume (20 ml) was orally administered to each pig, thereby delivering a total dose of 1.5 x 10^7 $TCID_{50}$.

In Study Two, the pigs were inoculated with material prepared using clinical specimens collected from PEDV-infected piglets on commercial farms. Clinical samples were tested by PEDV rRT-PCR to confirm the presence of the virus and then stored at -20 °C. To inoculate pigs with PEDV, field specimens were thawed at room temperature, mixed with 500 ml of

phosphate buffer saline (PBS, 1X pH 7.4, Sigma-Aldrich), and then sprayed into the nares of each pig for 5 sec using a garden sprayer (Chapin^{TM}, Batavia, NY). After inoculation, the inoculum was submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) for PEDV rRT-PCR testing and sequencing.

Sample collection

Serum

In Study One, blood samples (n = 132) were collected on DPIs -7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42. Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection system (Becton Dickson, Franklin Lakes, NJ) and serum separation tubes (Kendall, Mansfield, MA). Samples were then centrifuged (1,500 × *g* for 15 min), aliquoted into 2 ml cryogenic tubes (Greiner Bio-One GmbH, Frickenhausen, Germany), and stored at -80°C until tested. In Study Two, blood samples (n = 160) were collected on DPIs -4, 0, 7, 14, 21, 28, 35, and 42. Blood samples were drawn from the jugular vein or cranial vena cava using 12 ml disposable syringes (Covidien^{TM}, Minneapolis, MN) then transferred to serum separation tubes (Kendall, Mansfield, MA).

Oral fluid

To collect oral fluid, 3-strand, 1.6 cm, 100% cotton rope was hung from a bracket fixed to one side of each pen for 30 min, during which time the pigs chewed on and interacted with the rope. After 30 min, the wet end of the rope was severed, sealed in a plastic bag, and then passed through a clothes wringer (Dyna-Jet, Overland park, KS). The oral fluid that accumulated in the bottom of the bag was decanted into tubes for storage. In Study One, oral fluid specimens were collected twice daily (07:00 am and 1:00 pm) on DPI -3, 0, 5, 10, 15, 20, 25, 30, 35 and 42. The oral fluids collected at 07:00 each day were stored in a cooler until mixed with the oral fluids collected at 13:00, after which the cumulative sample was aliquoted into 2 ml cryogenic tubes (Greiner Bio-One GmbH) and stored at -80°C. In Study Two, oral fluid specimens were collected once daily (08:30 am) on DPIs -4, 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, and 42 and decanted into a 50 ml centrifuge tube (Fisher Scientific, Pittsburgh, PA) and stored at -20°C

Feces

In Study One, semi-solid fecal samples were collected from the floor of each pen on DPIs -4, 0 and 7. In Study Two, fecal samples were collected on DPIs -7, 0, and 7 using a separate sterilized fecal loop (VetOne®) for each individual pig. Samples were mixed with 1 ml of PBS (1X pH 7.4, Sigma-Aldrich) immediately after collection. All fecal samples were tested by PEDV rRT-PCR and the results used to establish the PEDV infection status of the inoculated pigs.

PEDV assays

PEDV rRT-PCR

Inoculums and fecal samples were tested for the presence of PEDV RNA by rRT-PCR using a procedure described by Madson et al. (2014) and performed routinely at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL SOP 9.5263). Primers and probes targeting conserved regions of the PEDV N gene were designed to match the US PEDV nucleotide sequences published in GenBank® (accession no. KF272920) (Madson et al., 2014).

In brief, total nucleic acids were extracted from samples using the MagMAX™ Pathogen RNA/DNA kit (Applied Biosystems, Life Technologies, Carlsbad, CA) and an automated 96-well magnetic particle processor (Thermo Scientific Kingfisher Flex, Thermo Fisher Scientific, Pittsburgh, PA) as per the manufacturers' instructions. The eluted RNA, primers, and probe were mixed with commercial reagents (Path-ID® Multiplex One-Step RT-PCR kit, Life Technologies) and the rRT-PCR reactions were conducted on an ABI 7500 Fast instrument (Life Technologies) as follows: 48°C for 10 min, 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 45 s. The results were analyzed using an automatic baseline setting with a threshold at 0.1. Quantification cycle (Ct) values < 35 were considered positive for PEDV.

PEDV spike (S) protein sequencing

The complete genome sequence of the PEDV isolate used in Study One (USA/IN/2013/19338E) has been reported elsewhere (Chen et al., 2016). In Study Two, the

part of the S1 gene (amino acid positions 1 to 744) of the PEDV in the inoculum was amplified and sequenced using the following primers: PEDVS1F (5-TTCTAATCATTTGGTCAACGTAAAC-3), and PEDVS1R (5-TACTCATACTAAAGTTGGTGGGAATAC-3). The RT-PCR products were purified using QIAquick PCR purification kit (Qiagen Co., Hilden, Germany) according to the manufacturer's protocol and then sequenced at the Iowa State University DNA facility. The S1 gene sequences were collated with PEDV sequences available through the National Center of Biotechnology Information (NCBI) using BLAST (https://blast.ncbi.nlm.nih.gov/). Thereafter, the sequences were aligned to other US PEDV strains in the NCBI database using the ClustralW alignment algorithm as described elsewhere (Chen et al., 2016). Sequence alignment was done by MEGA (v7.0, Pennsylvania State University, State College, PA). Sequence genomic identity was calculated from the aligned sequences by BioEdit (v7.2.5, Ibis Bioscience, Carlsbad, CA). A phylogenetic tree was constructed using the distance-based neighbor-joining (NJ) and maximum likelihood (ML) methods.

PEDV IgG and IgA ELISAs

Isotype-specific (IgG, IgA) PEDV ELISAs were based on isolate USA/NC35140/2013, as fully described elsewhere (Poonsuk et al., 2016). Following propagation on Vero cells, flasks underwent one freeze-thaw cycle, and then the harvested material was centrifuged at 4,000 × *g* for 15 min to remove cell debris. Thereafter, the virus was pelleted by ultracentrifugation at 140,992 × *g* for 3 h the virus pellet was washed twice with sterile PBS (1X pH 7.4), and then the purified virus was resuspended in PBS (1X pH 7.4) at a dilution of 1:100 of the original supernatant volume and stored at -80°C. Following titration and optimal dilution, the viral antigen solution was then manually coated (100 µl per well) onto 96-well polystyrene plates (Nunc® A/S, Rosklide, Denmark) and incubated at 4°C overnight in a closed container containing a towel saturated with water. After incubation, plates were washed 5 times, then the coated antigen was blocked with a blocking solution containing 1% bovine serum albumin (300 µl per well) (Jackson ImmunoResearch Inc., West Grove, PA). The blocked plates were incubated at 25°C for 2 h, then dried at 37°C for 4 h and stored at 4°C in a sealed bag with desiccant packs. Plate lots with a coefficient of variation ≥ 10% were rejected.

ELISA conditions for the detection of anti-PEDV IgA and IgG antibodies in serum and oral fluid were identical, i.e., coating and blocking conditions, reagent concentrations, incubation times, and buffers. Serum and oral fluid dilutions were adjusted to provide a uniform response across lots. High positive, low positive, and negative plate controls, i.e., antibody-positive and -negative experimental serum, or oral fluid specimens were run in duplicate on each ELISA plate. Serum samples were diluted 1:50 and oral fluid samples were diluted 1:1, after which plates were loaded with 100 µl of the diluted sample per well. Plates were incubated at 25°C for 1 h and then washed 5 times with PBST wash solution (PBS 1X , 0.1% Tween® 20, pH 7.4).

To perform the assay, 100 µl of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories Inc., Montgomery, TX) diluted 1:20,000 for serum, and 1:3,000 for oral fluid or goat anti-pig IgA (Bethyl Laboratories Inc.) diluted 1:3,000 for serum and 1:6,000 for oral fluid samples was added to each well and the plates incubated at 25°C for 1 h. After a washing step, the reaction was visualized by adding 100 µl of tetramethylbenzidine-hydrogen peroxide (TMB, Surmodics, Eden Prairie, MN) substrate solution to each well. After a 5 min incubation at room temperature, the reaction was stopped by the addition of 100 µl of stop solution (450 nm BioFX®, Surmodics) to each well. Reactions were measured as optical density (OD) at 450 nm using an ELISA plate reader (Biotek® Instruments Inc., Winooski, VT) operated with commercial software (GEN5™, Biotek® Instruments Inc.). The antibody response in serum, and oral fluid specimens were represented as sample-to-positive (S/P) ratios:

$$S/P\ ratio = \frac{(sample\ OD - blank\ well\ control\ mean\ OD)}{(positive\ control\ mean\ OD - blank\ well\ control\ mean\ OD)}$$

Bjustrom-Kraft et al. (2016) reported diagnostic sensitivities and specificities for serum and oral fluid PEDV IgG and IgA ELISAs across a range of S/P values. In this study, samples with S/P ratios ≥ 0.80 were considered positive for the PEDV serum IgG ELISA and for the oral fluid IgG and IgA ELISAs.

ELISA oral fluid clarification treatment formulations

Each oral fluid sample was divided into 4 aliquots. Each aliquot was treated with one of three clarification formulations (A, B, C), with the fourth aliquot serving as an untreated control (D). Formula A consisted of 100 ppm chitosan oligosaccharide lactate (Sigma), 0.1% polyethylene glycol sorbitan monolaurate (Tween® 20, Sigma), 0.5% bovine serum albumin (BSA) (Jackson immunoresearch, West grove, PA), and 1 ppm xylene cyanol in PBS (1X, pH 7.4). Formula B was identical to Formula A, minus Tween® 20. Formula C was identical to Formula A, except that it did not include chitosan oligosaccharide lactate.

To avoid diluting oral fluid samples, formulations were lyophilized in 5 ml round-bottom polystyrene tubes (Falcon®, Radnor, PA). For lyophilization, 1 ml of the formula was aliquoted into a tube, held at -80°C for 24 h, and then lyophilized (FreeZone™, Labconco®, Kansas city, MO) for 15 h. After lyophilization, tubes sealed with polyethylene snap-caps (Falcon®), and stored at room temperature in a vacuum-sealed plastic bag.

Prior to treatment, oral fluid specimens were thawed by holding at 4°C for 16 h in an environmental chamber (Caron®, Marietta, OH) and then 25°C for 2 h. Specimens were treated by adding 1 ml of each sample to one tube of each of the three formulations (A, B, C). Treated samples and the untreated control (D) were vortexed for 5 sec and then centrifuged at 1200 × *g* for 3 min at 4°C. The supernatant was then harvested and tested by PEDV IgA ELISA and PEDV IgG ELISA.

Effect of time on oral fluid and serum ELISA results

Immediately after initial testing, all oral fluid samples (n = 104) and a subset of serum samples (n = 38; Study Two, DPI 0, 42) were held at 4°C in an environmental chamber and retested on days post treatment (DPT) 2, 4, and 6. Samples were not vortexed or centrifuged prior to testing.

2.7 Data analysis

ELISA IgG and IgA results for serum (n = 38) and oral fluid (n = 104) samples were analyzed for the effect of chemical treatment (oral fluids) and time (serum and oral fluids) using commercial software (SAS® 9.4, SAS® Institute Inc., Cary NC, USA). The Shapiro-Wilk and Anderson-Darling tests (alpha level 0.05) rejected the assumption of normality for both serum and oral fluid datasets; therefore a nonparametric approach was utilized.

The effect of time on ELISA-detectable PEDV serum antibody was evaluated by comparing day 0 PEDV IgG and IgA ELISA S/P ratios to results generated after 2, 4, and 6 days of storage at 4°C using the Kruskal-Wallis test. For oral fluids, the effect of "clean-up" formulation (A, B, C), time (DPT 0, 2, 4, 6), and their interactions on PEDV IgA and IgG ELISA S/P ratios were evaluated by comparing treated sample S/P ratios to time-matched untreated control S/P ratios using the Kruskal-Wallis test. Treatment and DPT were analyzed as fixed effects and sample as a random effect. Thereafter, the Dwass-Steel-Critchlow-Fligner method was used to make pairwise comparisons between combinations of treatments and DPTs. Finally, the effect of "clean-up" formulation (A, B, C), time (DPT 0, 2, 4, 6), was evaluated separately for antibody-negative and antibody-positive samples. For this analysis, oral fluid samples collected prior to DPI 7 were considered negative and oral fluid samples collected after DPI 14 were considered positive.

Results

3.1 Clinical response to PEDV inoculation

In Study One, all pigs in the PEDV negative group were clinically normal throughout the experiment. Watery diarrhea was observed in PEDV inoculated pigs both in Study One and Study Two after inoculation. One animal was removed from Study Two due to vaginal prolapse and humanely euthanized by a qualified veterinarian.

3.2 PEDV rRT-PCR and sequencing results

The inoculums used in Study One and Study Two were positive for PEDV by rRT-PCR testing. Partial sequencing of the S1 gene identified the viruses in the inoculums as U.S.

non-S-INDEL PEDV, with 99% amino acid identity between Study One and Two viruses and 93% to a U.S. S-INDEL PEDV (USA/Iowa106/2013).

In Study One, pen fecal samples were collected from the floor of each pen on DPIs -4, 0 and 7. In Study Two, individual pig fecal samples were collected on DPIs -7, 0, and 7. In Study One, all fecal samples from the negative control group were PEDV rRT-PCR negative. All PEDV-inoculated pigs in Study One and Two were PEDV rRT-PCR negative at DPIs ≤ 0, but positive by DPI 7.

3.3 PEDV antibody ontogeny

Using a stringent approach, samples with S/P values ≥ 0.80 were considered positive in the serum IgG ELISA and the oral fluid IgG and IgA ELISAs [6]. As shown in Figure 1A, all serum samples were negative at DPIs ≤ 0 and all negative control animals (Study One) tested PEDV IgG ELISA negative throughout the study. All virus-inoculated pigs were serum PEDV IgG ELISA positive by DPI 14 and thereafter.

As shown in Figure 1B and C, all oral fluid samples collected from negative control pigs were negative by PEDV ELISA (IgG and IgA). Oral fluid samples from PEDV-inoculated pigs were PEDV IgG and IgA negative at DPIs ≤ 0, but positive for both IgG and IgA at DPIs ≥ 14.

3.4 Effect of chemical treatments and time (4°C) on PEDV IgG, IgA ELISAs

The lyophilized formulations (A, B, C) were quickly dissolved by the addition of the oral fluid, i.e., the formulations were compatible with the lyophilization process. The effect of chemical treatment on the appearance of oral fluid is shown in Figure 2. Oral fluid treated with formulations A or B were more translucent than controls or oral fluid treated with formulation C.

Tables 1 and 2 summarize the effect of treatment and time on PEDV IgG and IgA ELISA S/P ratios for serum and oral fluids. Analysis of serum IgG and IgA S/P ratios from samples held at 4°C and tested at DPT 0, 2, 4, 6 did not detect a significant effect of time on the results (*p*

> 0.05). Further, pairwise comparisons of DPT 0 versus DPT 2, 4, and 6 results detected no differences ($p > 0.05$) in serum IgG or IgA ELISA S/P ratios over time. Analysis of oral fluid IgG and IgA S/P ratios found that neither chemical treatment of the sample nor time held at 4°C significantly affected the results ($p > 0.05$). Pairwise comparisons of DPT 0 to DPT 2, 4, and 6 results detected no differences ($p > 0.05$) in oral fluid IgG or IgA ELISA S/P ratios over time.

Discussion

Coagulation is the process of destabilizing the electrical charges on the surface of particles suspended in liquids; thereby allowing the particulates to aggregate (flocculate) and then precipitate out of solution (Bratby 1981; Bratby, 2006a; Bratby, 2006b). Coagulants, the chemicals that initiate the process of coagulation, have a long history. For example, alum (hydrated potassium aluminum sulfate) was used to clarify water in Egypt as early as 1500 BCE (Angelakis et al., 2012). Today, coagulants are used in diverse industrial and agricultural applications because they are readily available, inexpensive, fast-acting, and safe.

Coagulants are chemically highly diverse and include inorganic metal salts (alum, potassium ferrate, ferrous sulfate, ferric chloride, magnesium chloride, and polyaluminium chloride), synthetic polymers (hexa-methylene diamine epichlorohydrin polycondensate, polyethyleneimine, polytrimethyl ammonium chloride) and organic polymers (chitosan, cellolose, and sodium algenate) (Verma et al., 2012; Lee et al., 2014). Chitosan, the coagulant evaluated in this research, is a polysaccharide made from chitin derived from the exoskeletons of crustaceans, e.g., shrimp and lobsters (Benjakul and Sophandora, 1993; Weska et al., 2007). Several chemical forms of chitosan have been described. These derivatives are uniformly non-toxic, non-allergenic, and biodegradable (Thanou et al., 2001). Chitosan or chitosan-based nanoparticles have been evaluated in a variety of biological or medical applications, e.g., livestock feed supplement (Hirano et al., 1990; Illum, 1998), wound dressings (Hoekstra et al., 1998; Khan et al., 2000; Wedmore et al., 2006), drug delivery (Agnihotri et al., 2004Dev et al., 2010), vaccines (van der Lubben et al., 2001) and cancer diagnostics (Jayakumar et al., 2010). Likewise, chitosan has been used in a variety of

environmental applications e.g., removal of metal ions, viral/bacterial pathogens, and suspended particulates from drinking water (Elson et al., 1980; Jagtap et al., 2011; Kamble at al., 2007), surface water (Huang and Chen, 1996; Rizzo et al., 2008), and industrial or agricultural waste water (Boddu et al., 2003; Bhatnagar and Sillanpää, 2009; Saifuddin and Kumaran, 2005). In environmental applications the concentration of chitosan ranges from 0.5 to 4,000 ppm, depending on the application (Ishii et al., 1995; Kim and Rajapakse, 2005).

The objective of this experiment was to evaluate the use of chitosan for the clarification of swine oral fluid samples. Three formulations (A, B, C) plus an untreated control (D) were evaluated. A blue dye (xylene cyanol, 1 ppm) was added to each formulation in order to readily identify treated oral fluid samples. BSA (0.5%) was added to all formulations to block nonspecific reactions, i.e., improve the specificity of the antigen-antibody reactions (Steinitz, 2000). Formulations A and C contained Tween-20® (0.1%), a detergent known to block unoccupied protein binding sites and dissolve unstable hydrophobic bonds (Batteiger et al., 1982; Steinitz, 2000). However, detergents can also adversely affect the attachment of proteins to polystyrene ELISA plates and/or interfere with antibody binding when used above the optimal working range (0.05%) (Engvall and Pearlmann, 1972; Hoffman and Jump, 1986; Julián et al., 2001; Gardas and Lewartowska, 1988). One detergent-free treatment (formulation B) was included to detect this response. Formulations A and B contained chitosan (100 ppm); a level consistent with water treatment applications (Chi and Cheng, 2006). The inclusion of chitosan at 100 ppm was found to effectively clarify oral fluids (Figure 1). Formulations A, B, and C were lyophilized to avoid diluting the samples, minimize environmental contamination, improve stability, and to anticipate the use of this process in the field. This approach was effective, i.e., all formulations went into solution quickly and easily upon addition of oral fluid.

None of the treatments affected PEDV IgG or IgA ELISA S/P ratios, either in the short term (DPT 0) nor over time (DPT 2, 4, 6) when compared to untreated controls (D). The absence of a residual effect of treatment is important because it implies that the treatment could be applied in the field. Likewise, it suggests that veterinary diagnostic laboratories could safely store treated oral fluid samples at 4°C for a short time without affecting testing results.

This study found that chitosan could efficiently clarify oral fluid specimens without affecting the results of PEDV antibody ELISAs. Future work should verify the applicability of these results to other pathogens and other diagnostic technologies. Given the variety and number of available coagulants, it would also be reasonable to speculate that other coagulants or formulations could provide equal or better results. Thus, this study should be interpreted as the initiation of a new line of research in oral fluid diagnostics.

Declaration of conflicting interests

The author(s) declare that there are no conflicts of interest with respect to their authorship and/or the publication of this manuscript.

Acknowledgments

The authors thank Drs. Qi Chen, Alexandra Henao, Ting-Yu Cheng, Juan Carlos Mora, Melisa Spadaro, and Elisa Gibert for technical assistance with animal experiments and/or laboratory assays. This work was funded in part by Pork Checkoff Funds distributed by the Iowa Pork Producers Association through the National Pork Board (#15-142; Des Moines, Iowa 50325), Zoetis Animal Health (Parsippany, New Jersey 07054), and the Iowa State University Veterinary Diagnostic Laboratory (Ames, Iowa 50011)

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Tables

Table 1. Effect of treatment and time[a] on PEDV IgG S/P ratios (mean, SD)

Specimen and treatment	Day post treatment			
	Day 0	Day 2	Day 4	Day 6
Untreated oral fluid[b]				
Negative	0.0 (0.1)	0.0 (0.1)	0.0 (0.0)	0.0 (0.0)
Positive	1.4 (1.2)	1.5 (1.3)	1.4 (0.1)	1.1 (0.9)
Oral fluid treatment A[b]				
Negative	0.0 (0.1)	0.0 (0.1)	0.0 (0.1)	0.0 (0.1)
Positive	1.0 (0.9)	1.0 (1.0)	1.1 (1.0)	0.9 (1.1)
Oral fluid treatment B[b]				
Negative	0.0 (0.1)	0.0 (0.1)	0.0 (0.1)	0.0 (0.1)
Positive	1.2 (1.1)	1.4 (1.3)	1.3 (1.1)	1.1 (0.8)
Oral fluid treatment C[b]				
Negative	0.0 (0.1)	0.0 (0.1)	0.0 (0.1)	0.0 (0.1)
Positive	1.0 (0.9)	1.1 (1.0)	1.0 (1.0)	0.9 (0.9)
Serum (no treatment) [b]				
Negative	0.1 (0.2)	0.1 (0.2)	0.1 (0.2)	0.1 (0.2)
Positive	1.9 (0.5)	1.8 (0.5)	1.7 (0.5)	2.2 (0.7)

[a]Samples held at 4°C

[b]Pairwise comparisons of DPT 0 IgG S/P ratios to DPT 2, 4, and 6 results detected no differences in IgG ELISA S/P ratios over time (nonparametric ANOVA, $p > 0.05$).

Table 2. Effect of treatment and time[a] on PEDV IgA S/P ratios (mean, SD)

Specimen and treatment	Day post treatment			
	Day 0	Day 2	Day 4	Day 6
Untreated oral fluid				
Negative	0.0 (0.1)	0.0 (0.1)	-0.1 (0.1)	-0.1 (0.1)
Positive	1.5 (1.1)	1.4 (1.0)	1.4 (1.0)	1.4 (1.1)
Oral fluid treatment A[b]				
Negative	0.0 (0.1)	0.0 (0.1)	-0.1 (0.1)	-0.1 (0.1)
Positive	1.3 (0.9)	1.2 (0.9)	1.2 (0.9)	1.2 (1.0)
Oral fluid treatment B[b]				
Negative	-0.1 (0.1)	-0.1 (0.1)	-0.1 (0.1)	-0.1 (0.1)
Positive	1.3 (1.0)	1.3 (0.9)	1.2 (1.0)	1.3 (1.0)
Oral fluid treatment C[b]				
Negative	0.0 (0.1)	-0.1 (0.1)	-0.1 (0.1)	-0.1 (0.1)
Positive	1.3 (0.9)	1.2 (0.8)	1.2 (0.9)	1.2 (0.9)
Serum (no treatment) [b]				
Negative	0.1 (0.1)	0.1 (0.1)	0.1 (0.2)	0.1 (0.1)
Positive	2.7 (1.1)	2.4 (1.1)	2.6 (1.3)	2.3 (1.0)

[a]Samples held at 4°C

[b]Pairwise comparisons of DPT 0 IgA S/P ratios to DPT 2, 4, and 6 results detected no differences in IgA ELISA S/P ratios over time (nonparametric ANOVA, $p > 0.05$).

Figures

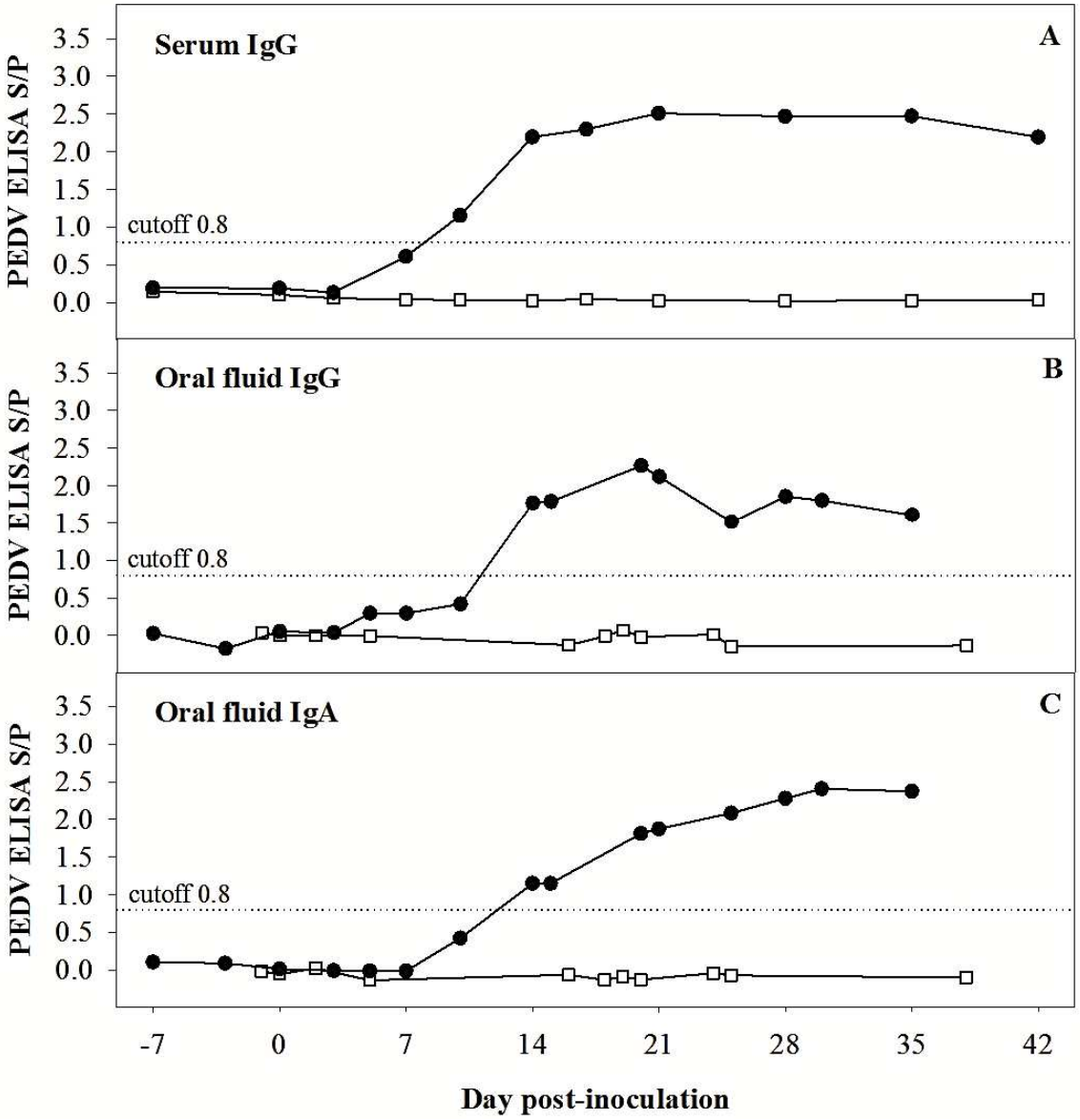

Figure 1. PEDV isotype-specific antibody (mean ELISA S/P response) by day post inoculation in serum and oral fluid specimens collected from PEDV-inoculated (●) and negative control (□) groups.

Figure 2. Oral fluid specimens following treatment. NC = negative control; A = formulation A (chitosan 100 ppm, Tween-20® 0.1%, BSA 0.5%, xylene cyanol 1 ppm); B = formulation B (chitosan 100 ppm, BSA 0.5%, xylene cyanol 1 ppm); C = formulation C (Tween-20® 0.1%, BSA 0.5%, xylene cyanol 1 ppm)

CHAPTER 6. GENERAL DISCUSSION

Three components of lactogenic immunity play a key role in protecting neonates against infectious agents until their immune systems are fully developed: (1) circulating antibodies derived from colostrum, (2) mucosal antibodies from colostrum and milk, and (3) immune cells provided in mammary secretions (Salmon et al., 2009). To a large extent, pioneering work in transmissible gastroenteritis virus (TGEV) revealed the biological processes underlying lactogenic immunity. Enteric TGEV replication is needed to stimulate the plasmablasts in the intestinal lamina propria that migrate to mammary gland parenchyma where they produce TGEV antibodies (Bohl et al., 1972; Saif et al., 1972). These antibodies accumulate in mammary secretions and are delivered to the suckling piglet. Lactogenic antibody is protective both within the intestinal tract of the neonatal pig and in the circulatory system of the piglets (Song et al., 2007; Song et al., 2015).

To a large extent, the concepts of lactogenic immunity developed through TGEV research can be transferred to the prevention and control of PEDV. As is true for TGEV, it has been observed that prior exposure of the sow herd to PEDV ameliorates the impact of the infections in neonates (Goede and Morrison, 2016). Therefore, to protect piglets against clinical PED, intentional exposure and/or vaccination have been used in the field to stimulate lactogenic immunity and protect neonatal pigs against clinical disease.

The research described in this dissertation further clarified the role of lactogenic immunity in the protection of neonates against PEDV and provided further evidence that lactogenic immunity is vital to the protection of the neonatal pig against PEDV and that the optimal protection to piglets will be provided by dams able to deliver sufficient lactogenic immunity to their offspring. Thus, S-IgA is central to limiting the replication of PEDV in the intestinal tract and circulating antibody is protective against clinical disease (Poonsuk et al., 2016a; Poonsuk et al., 2016b).

However, beyond these core facts, significant questions related to PEDV immunology and the prevention/control of clinical PEDV remain to be answered, e.g., *What level of maternal*

immunity (both humoral and cell-mediated) is protective? How can we efficiently measure and predict protective levels of lactogenic immunity? How can we effectively manipulate lactogenic immunity sufficient to the protection without the use of live virus? And especially, *How can we quantify or measure lactogenic immunity prior to parturition?*

Thus, important questions remain to be answered, but future research on PEDV lactogenic immunity should focus on two areas: (1) the development of quantitative assays able to provide predictive measures of lactogenic immunity; and (2) effective approaches to stimulate protective levels of lactogenic immunity in a sow herd without the use of live virus.

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ACKNOWLEDGEMENTS

I am deeply indebted to my major professor, Dr. Jeffrey Zimmerman, for providing me opportunities, generous support and guidance throughout my graduate studies and also future.

I am very grateful to my program of study committee: Dr. Jianqiang Zhang, Dr. Roger Main, Dr. Chong Wang, and Dr. Darin Madson for being always willing to support me. All your guidance, comment, and suggestions have been valuable in improving my research and this dissertation.

I would like to thank Drs. Luis Giménez-Lirola for his friendship and mentorship on my study. I extend my sincere thanks to my friends in the swine ecology laboratory: Dr. Wendy Gonzales, Dr. Marisa Rotolo, Dr. Kevin O'Neill, Dr. Melisa Spadaro, Dr. Alexandra Henao-Díaz, Dr. Ting-Yu Cheng, Dr. Luciana Sarmento, and Dr. Juan Carlos Mora for their friendship and their assistance. I would like to thank Dr. Roongroje Thanawongnuwech for his advice and inspiration during my graduate studies.

Lastly, I would like to thank my family for their love and patient support during this challenging time.